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REMARKS

Claims 1-17 are pending in the application. Applicants have amended claims 1, 11, and 13. Claim 12 has been canceled.

Upon entry of the present amendment, claims 1-11 and 13-17 remain pending in this application.

Claims 1-17 stand rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. Specifically, the recitation of the limitations of (C₁-C₆)alkyl was thought to be unclear.

According to the second square bullet, R² may indeed be (C₁-C₆)alkyl substituted with OC(=O)R^{4a}, NR-SO₂-R³, RN-SO₂-NR^{4a}R^{5a} or NR-CO-NR^{4a}R^{5a}.

According to the first square bullet, R² may also be (C₁-C₆)alkyl substituted with one to three OR⁴, COOR⁴, NR⁴R⁵, NRC(=O)R⁴, C(=O)NR⁴R⁵ or SO₂NR⁴R⁵. However, in these definitions, R⁴ is (C₁-C₆)alkyl which is further substituted. Therefore, the groups envisaged by the Examiner are, in fact, O-alkyl-C(=O)R⁷, N(R⁵)-alkyl-SO₂R⁶, N(R⁵)-alkyl-SO₂NR⁷R⁸ and N(R⁵)-alkyl-C(=O)NR⁷R⁸. Applicants respectfully submit that these definitions do not overlap with those of the second-square bullet.

Claim 11 was specifically rejected as it was allegedly unclear if species were being claimed or a mixture of some (or all) species were being claimed. Applicants have amended claim 11 to overcome this rejection.

Claims 2-10 and 12-17 were rejected as being dependent on claim 1. Applicants have amended claim 1 thereby rendering moot the objection to these claims.

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Accordingly, Applicants respectfully submit that all of the rejections to the claims under 35 U.S.C. § 112, second paragraph, have been overcome and reconsideration of the rejections is respectfully requested.

Claims 12-16 stand rejected under 35 U.S.C. § 112, first paragraph, because as stated in the Office Action, the specification, while being enabling for the treatment of AIDS (or HIV infection), allegedly does not reasonably provide enablement for the treatment of other diseases such as: T-cell related diseases, autoimmune diseases, osteoarthritis, rheumatoid arthritis, multiple sclerosis, osteoporosis, chronic obstructive pulmonary disease (COPD), asthma, cancer, leukemia, allergy, inflammatory bowel disease (IBD), ulcerative colitis, Crohn's disease, pancreatitis, dermatoses, psoriasis, atopic dermatitis, glomerulonephritis, conjunctivitis, autoimmune diabetes, graft rejection, epilepsy, muscular atrophy and systemic lupus erythematosus. It is further alleged in the Office Action that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with the claims.

At the outset, the Applicants acknowledge and appreciate the Examiner's indication that the treatment of AIDS (or HIV infection) is enabled by the specification. Applicants respectfully submit that the following diseases/conditions are also enabled by the specification: allergy, asthma, T-cell mediated disease, dermatoses (atopic dermatitis), cancer (leukemia), and osteoporosis.

The Applicants respectfully contend that claims 12-16 are enabling with respect to the full scope of the claims; however, in order to advance the prosecution of the present invention Applicants have amended claim 13. Applicants reserve their right to file a divisional application directed to the canceled subject matter in due course.

Upon review and consideration of the rejections set forth in the office action, Applicants contend that the specification enables any person skilled in the art to which the invention pertains, or with which it is most nearly connected, to use the invention commensurate in scope with the amended claim 13.

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Applicants respectfully submit that one skilled in the art would find the asserted utility of the claimed compounds consistent with the knowledge in the art at the time of the filing of the application covering the present invention. The scientific literature establishes a direct relationship between PDE7 inhibition and the claimed utilities. To establish this relationship the following literature references are provided below in Table 1, which describes the association between the mechanism of the claimed compounds and the claimed utilities.

Table I

Claimed Disorder	Literature Reference	Disorder in Literature Reference
Allergy and asthma:	Schudt, C. et al, Pulmonary Pharmacology and Therapeutics (1999) 12, 123-129	This paper provides a potential link between PDE7 and asthma, and presents data in a table (table 2) showing the expression of PDE7 in human cells (including endothelial cells).
	Fuhrmann, M. et al, Am. J. Respir. Cell Mol. Biol. (1999) 20; 292-302	This paper shows the expression of PDE7 mRNA in human and porcine airway epithelial cells (figures 1-3) but it is difficult to characterise the protein due to the fact that no antibodies or inhibitors were known at the time. But this paper does provide a potential link between PDE7 and airway inflammation (e.g. asthma, COPD).
	Wright, L. et al AJP Lung Cell Mol. Physiol. 275; L694-L700 (1998)	This paper shows the expression of PDE7 mRNA in human epithelial cells (figure 5) and hence provides a link between PDE7 and airway inflammation.
T-cell mediated disease:	Kanda, N. and Watanabe, S. Biochemical Pharmacology (2001), 62, 495-507	This paper shows that PDE7 antisense DNA blocks PDE1,2,3,4-independent activity induced by phytohemagglutinin or

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Claimed Disorder	Literature Reference	Disorder in Literature Reference
		anti-CD3 plus anti-CD28 in T-cells. Furthermore, PDE7 mRNA is increased in stimulated T cells (stimulated by anti-CD3 plus anti-CD28). The paper therefore provides a link between PDE7 and T-cell mediated disease.
	Li, L. et al Science (1999) 283; 848-851	This paper shows the induction of PDE7 and the consequent suppression of cAMP-dep-PKA activity is required for T-cell activation. Therefore, they postulate that PDE7 inhibitors could be an approach for treating T-cell dependent disorders.
	Nakata, A. et al Clin Exp Immunol. (2002) 128; 460-466	This paper links PDE7 to T-cell mediated diseases (specifically immunological and inflammatory disorders - e.g. asthma, allergic dermatitis) and they show that a PDE7 inhibitor (T-2585) decreases IL-5 synthesis, decreased proliferation and decreased CD25 expression in peripheral blood mononuclear cells (PBMC). (IL-5 is a key inflammatory cytokine.)
Dermatoses - atopic dermatitis:	Gantner, F. et al British Journal of Pharmacology (1998) 123; 1031-1038	This paper shows PDE7 activity and mRNA at high levels in normal B-cells (from non-atopic patients) and in atopic B-cells (patients with atopic dermatitis) (figure 1). This therefore provides a link between PDE7 and dermatoses.

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Claimed Disorder	<u>Literature Reference</u>	<u>Disorder in Literature Reference</u>
Cancer – leukaemia:	Lee, R. et al Cellular Signalling (2002 – April) 14 277-284	This paper provides a link between PDE7 and leukemia. They show PDE7 protein expression in normal B-cells, primary chronic lymphocytic leukemia (CLL) cells and in a CLL-derived cell line (WSU-CLL) and PDE7 levels were augmented by treatment of these cells with methylxanthines (non-specific PDE inhibitor).
Osteoporosis:	Wakabayashi et al Journal of Bone and Mineral Research (2002) 17; 249-256	This paper shows PDE7 mRNA expression in bone osteoblastic cell lines and therefore provides a link between PDE7 and osteoporosis.

Applicants respectfully submit that the literature references provided herewith, together with other literature cited in the application, provide guidance to one of skill of the art for a link between PDE7 inhibitors of the present invention and the claimed diseases/disorders.

As a courtesy to the Examiner, copies of the literature references discussed above are attached hereto.

Accordingly, Applicants submit that the rejection of claims 12-16 (now claims 13-16) under 35 U.S.C. § 112, first paragraph, has been overcome. Applicants respectfully request that the rejection be withdrawn.

Claims 1-17 stand provisionally rejected under the judicially created doctrine of obviousness – type double patenting as allegedly being unpatentable over claims 1 and 3-26 of co-pending application 10/852,404 (or US2004/021483 A1). It is alleged in the Office Action

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that the instant formula (I) overlaps with the formula (I) of the co-pending application when variables of the co-pending application have the following meanings:

- i. X_1 - X_4 are independently $-C(R^1)-$; wherein R^1 is alkyl (which corresponds to the instant R^1) or R^1 is X^2R^3 (which corresponds to the instant $-OR^2$);
- ii. Y is NR^{12} wherein R^{12} is hydrogen;
- iii. A is a ring of 4-, 5- or 7-membered ring with ring atoms of A^1 , and/or A^2 , A^4 , A^5 ;
- iv. Z is O.

Further, it is alleged that "[c]laim 1 of the co-pending application differs from the instant claim 1 by having a broader scope and reciting formulae II & III. However, regarding Formula I, there is substantial overlapping subject matter as listed above. Further more, two species in claims 22 and 23 of the co-pending applications (see US 2004/214843 A1 page 66 left column, lines 49 and 52; also page 67, right column, lines 10 and 13) read on the instant Formula (I)".

Applicants respectfully submit that there is no overlap between the claims of US 2004/214843 A1 and the claims of the present application for the reason set forth immediately below. First, the claims of US 2004/214843 A1 define that the at the 5'-position (the group X_1) may be X^4R^4 or Q1. The group Q1 may be OR^2 .

Further, in the '843 application, the R^5 is restricted to aryl, heteroaryl, cycloalkyl (optionally incorporating C=O or a heteroatom) or cycloalkenyl (optionally incorporating C=O or a heteroatom). These groups are not included in the definition of R^2 in the present application.

In the alternative, the group R^2 , in the US 2004/214863 A1 may be a lower alkyl which may be further substituted with, among other things, OR^6 , $COOR^6$, NR^6R^7 , $NR^6C(=O)R^7$, $C(=O)NR^6R^7$ or $SO_2NR^6R^7$.

In the claims of the presently pending application, the equivalent group R^2 is defined as (C_1-C_6) alkyl which is further substituted with OR^4 , $COOR^4$, NR^4R^5 , $NRC(=O)R^4$, $C(=O)NR^4R^5$

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or $\text{SO}_2\text{NR}^4\text{R}^5$, and R^4 and R^5 are in turn, $(\text{C}_1\text{-C}_6)\text{alkyl}$ which is further substituted (in the case of R^4) and may be further substituted (in the case of R^5). Therefore, it can be seen, that the groups R^4 and R^5 in the claims of the presently pending application are equivalent to the groups R^6 and R^7 in US 2004/214863 A1.

However, the substituents on the groups R^4 and R^5 in the claims of the present application are different than those on the groups R^6 and R^7 in US 2004/214863 A1. In US 2004/214863 A1, the groups R^6 and R^7 may be hydrogen or lower alkyl which may be substituted with one to three OR, COOR or $\text{NR}^{23}\text{R}^{24}$ wherein R, R^{23} and R^{24} may be hydrogen or lower alkyl (or in the case of R, additionally CN or SO_2NH_2). In the claims of the present application, the substituents on the groups R^4 and R^5 do not include OR, COOR or $\text{NR}^{23}\text{R}^{24}$. Additionally, as a substituent must be present on the group R^4 in the present application, the possibility in the present application of R^4 being unsubstituted alkyl is excluded. There is, therefore, no overlap between these groups in the present application and those of US 2004/214863 A1.

In the alternative, as outlined above, the group R^2 in the present application may be $(\text{C}_1\text{-C}_6)\text{alkyl}$ which must be substituted with $\text{OC}(=\text{O})\text{R}^{4a}$, SR^{4a} , $\text{S}(=\text{O})\text{R}^3$, $\text{C}(=\text{NR}^5)\text{R}^{4a}$, $\text{C}(=\text{NR}^5)\text{-NR}^{4a}\text{R}^{5a}$, $\text{NR-C}(=\text{NR}^5)\text{-NR}^{4a}\text{R}^{5a}$, NR-COOR^{4a} , $\text{NR-C}(=\text{O})\text{-NR}^{4a}\text{R}^{5a}$, $\text{NR-SO}_2\text{-NR}^{4a}\text{R}^{5a}$, $\text{NR-C}(=\text{NR}^5)\text{-R}^{4a}$ or $\text{NR-SO}_2\text{-R}^3$. These substituents are not present in the list of possible substituents for the equivalent group R^2 in the US 2004/214863 A1. Therefore, there is no overlap between these groups in the claims of the present application and those of US 2004/214863 A1.

It is stated in the Office Action that the compound cited on page 66, left column left column, lines 49 and 52 (corresponding to Examples 81 and 82) are within the scope of the present application. Applicants respectfully submit that these compounds fall outside the scope of the present application for the reasons set forth immediately below. First, in Example 81 of US 2004/214863 A1, the group X_1 is $\text{C-OCH}_2\text{CN}$. This falls outside the present claims as CN is not included in either list of the substituents on the $(\text{C}_1\text{-C}_6)\text{alkyl}$ group immediately attached to the oxygen atom (see the two square bullets).

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Secondly, in Example 82, the group X_1 is $C-OCH_2-(1H-tetrazol-5-yl)$. This also falls outside the claims of the present application wherein the group Q^2 is a saturated heterocycle, not a heteroaryl group such as tetrazolyl.


Accordingly, Applicants respectfully submit that the claims 1-17 are patentably distinct from the claims of US 2004/214863 A1 and respectfully request that the rejection of claims 1-17 under the judicially created doctrine of obviousness – type double patenting be removed.

In view of the present amendment and forgoing remarks reconsideration of the rejection and advancement of the case to issue are respect fully requested.

The Commissioner is authorized to charge any fee or credit any over payment in connection with this communication to our Deposit Account No. 23-0455.

Respectfully submitted,

Dated: 8/16/05


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PULMONARY
PHARMACOLOGY
& THERAPEUTICS



Therapeutic Potential of Selective PDE Inhibitors in Asthma

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PKA AND cAMP AS SUPPRESSORS OF ACTIVATED CELLS

Cellular cyclic AMP (cAMP) concentrations and the corresponding activation state of protein kinase A are main regulators of signal transduction pathways. Various regulating proteins with bottleneck function such as ion channels, ion pumps and protein kinases are targets for phosphorylation and activity modulation by protein kinase A. Thereby cellular cAMP levels may serve as a gate or a valve regulating cellular functions and susceptibility for activating signals.^{1,2}

Prominent cellular functions associated with asthma are (1) leukocyte activation and proliferation, (2) transendothelial permeability and (3) contraction and proliferation of smooth muscle cells which can all be down-regulated by activated protein kinase A. Therefore, it must be inferred that increases of cellular cAMP levels should interfere with airway inflammation, or edema formation, bronchoconstriction and remodelling in asthma. Since actual cAMP concentrations are determined both by the rates of synthesis and breakdown, the cellular profile, and localization of PDE isoenzymes are important determinants of cAMP levels and consequently the activation state of cells involved in airway disorders. Typical deviations such as increased smooth muscle tone, enhanced sensitivity to allergens and airway inflammation as well as long-term airway remodeling may thus be normalized by selective inhibitors of PDE isoenzymes.

PDE SUPERFAMILY

In addition to the PDE6 isoenzyme involved in photo-receptor signaling at least 15 genes in the human genome code for more than 30 PDE proteins (splice variants). The PDE superfamily has been divided into nine families on the basis of genetic criteria, substrate

specificity, biochemical regulation and pharmacological properties as listed in Table 1. In view of this large superfamily of PDEs several issues have to be resolved in order to design new drugs which (1) specifically target cells central to the pathology of the respective disease (2) reverse pathophysiological deviations in cells involved and (3) do not simultaneously interact with other cells to minimize the risk of adverse drug reactions.

PDE ISOENZYME PROFILES

One possible approach to resolve the distribution of PDE isoenzymes in human cells and tissues makes use of (1) specific substrates, (2) the physiological regulators Ca/calmodulin and cGMP and (3) isoenzyme-selective drugs. This procedure identifies PDE isoenzymes in homogenates and fractions of purified human cells and quantifies the amount of PDE1–5 and PDE7 at the level of activity. Most of these data have been published,³ and are summarized in Table 2. PDE7 was not analysed directly but the remaining activity which could not be inhibited by a combination of selective inhibitors was attributed to this PDE family in accordance to PCR data. Any further differentiation of subtypes and splice variants has been studied in several tissues but conclusive – and especially semiquantitative – results related to human cells are scarce. However, such investigations are currently performed using techniques such as PCR and Western blot analysis.

Three groups of cells and tissues may be classified from the data in Table 2:

- (1) cells exclusively or predominantly containing PDE4 such as eosinophils, neutrophils, monocytes, B lymphocytes and epithelial cells. The latter in addition containing PDE1.
- (2) Mast cells and T cells which contain predominantly PDE4 and PDE3. Macrophages and dendritic cells containing PDE4, PDE3 and additional PDE1. Endothelial cells containing PDE4, PDE3 and additional PDE2.

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Table 1 Human phosphodiesterases.

Isoenzyme	Biochemical feature	(Specific) inhibition by (IC_{50} , μ M)	Substrate (K_m , μ M)	Genes	Splice variants
1	Ca^{2+} /CaM-stimulated	-	cGMP > (A, B) or = (C) cAMP	A B C	2 (>) 1 (>) 3 (>)
2	cGMP-stimulated	EHNA (1)	cAMP = cGMP (50)	A	1 (>)
3	cGMP-inhibited	Metapaxone (0.03) and many others	cAMP = cGMP (0.2)	A B	2 1
4	-	RP73401 (0.001) and many others	cAMP (5)	A B C D	2 3 4 (>) 5
5	cGMP binding	Sildenafil (0.003) and many others	cGMP (1)	A	1
6	'Photoreceptor' cGMP binding	-	cGMP	α , α , β , γ (8, 13 kD)	
7	Mg^{2+} -independent	IBMX (30)	cAMP (0.2)	A	2
8	-	Dipyridamole (10-40); not IBMX	cAMP (0.05)	A B	1 1
9	No cGMP binding	SCH51866 (1.5); Zaprinast (35)	cGMP (0.1)	A	1

- (3) Smooth muscle tissue from bronchi and *A. pulmonalis* containing PDE1, PDE3, PDE4 and PDE5 at characteristic amounts and ratios with bronchi containing additional PDE2.

These PDE isoenzyme profiles and activity ratios appear to be characteristic for the described cells and tissues after isolation or preparation. However, they can obviously be altered by a variety of exogenous or endogenous stimuli. For example, increased expression of PDE4 subtypes by β_2 -adrenoceptor agonists, prostaglandins and other cAMP-elevating agents was found in cardiac tissue,⁴ inflammatory cells,⁵ or keratinocytes,⁶ and this up-regulation in response to prolonged protein kinase A activation seems to be a general principle resulting in homologous or heterologous desensitization which may be of relevance in asthma.⁷

Cell differentiation and proliferation seems to be often accompanied by an alteration of PDE profile as shown for human T lymphocytes¹⁴ or smooth muscle cells.⁸ Data in Table 2 show details of another example of PDE isoenzyme changes during differentiation. Human monocytes which were differentiated under conditions of tissue culture to macrophages or dendritic cells adopted a new PDE isoenzyme profile. This was similar to the profile found in macrophages isolated from bronchoalveolar lavage and was characterized by a considerable increase of PDE1 and PDE3 paralleled by a decrease of PDE4.^{10,11} Thus, the transient, tissue-specific up-regulation of certain members of the PDE superfamily may be a

tool to regulate cellular sensitivity for extracellular signals.¹²

Several studies addressed the question of whether PDE isoenzyme profiles in PBMCs obtained from allergic subjects differ from those of normals. Conflicting results have been published and are reviewed by Gantner et al.¹³ Our own results suggested that there is no difference in PDE profiles of PBMC or eosinophils obtained from normal versus allergic volunteers.¹³ However, based on published effects of inflammatory mediators such as LPS, IFN- γ , IL-4, IL-10, PGE₂ or endogenous catecholamines on the PDE isoenzyme content of human monocytes or T cells,¹⁴⁻¹⁸ it may be speculated that cells which migrated from the blood compartment into areas of inflammation after being in contact with these pro-inflammatory mediators exhibit changes in their PDE isotype expression.

SELECTIVE INHIBITION OF PDE ISOENZYMES AND CORRESPONDING INFLUENCE ON CELL FUNCTIONS

Activation of the cells listed in Table 2 can be experimentally suppressed by addition of either forskolin or mediators such as PGE₂ or by dibutyryl-cAMP and other direct activators of protein kinase A.¹⁰⁻¹⁴ On the other hand, inhibition of cellular functions by cAMP agonists was reversed by PKA inhibitors. These results indicate that the influence of cAMP elevation

Table 2 PDE isoenzyme profiles of human cells.

	1	2	3	4	5	7	Reference
Eos, neutrophils	-	-	-	++++	-	++	19, 20
Monocyte	-	-	+	++++	+	++	10
MP; DC (mono-der)	++++	-	++++	++++	+	++	10
MP (BAL)	++++	-	++++	++++	+	++	11
Mast cell	+	-	+++	+++	+	ND	3
T cells	+	-	++	++	-	+	26
B cells	+	-	-	+++	-	+	48
Endothelial cells	+	+++	+++	+++	-	+	3
Epithelial cells	++	+	+	+	-	+	3
Platelets	+	++	+	-	+++	ND	3
<i>A. pulmonalis</i>	+	-	++++	+++	++++	ND	3
Bronchi	+	+	+++	+++	+++	ND	29

PDE activity (pmol/mg × min): <2-2.5 +, 5-15 ++, 15-45 +++, >45 ++++.

MP = macrophages. DC = dendritic cells. mono-der = monocyte-derived. BAL = broncho-alveolar lavage. ND = not determined.

Eos = eosinophils.

in most cases seems to be mediated by protein kinase A resulting in a suppression of cell activation. The necessary extent of specific PDE isoenzyme inhibition required for sufficient PKA activation that attenuates cellular functions can now be assessed.

EOSINOPHILS, NEUTROPHILS AND MONOCYTES

Induced release of reactive oxygen species (ROS) and leukotrienes by activated eosinophils,¹⁹ or neutrophils,²⁰ was suppressed by selective PDE inhibitors rolipram or piclamilast and the efficiency could not be further enhanced by PDE3 inhibitors such as motapizone. In monocytes, LPS-induced release of TNF- α was inhibited by maximum PDE4 inhibition to approximately 80%, and only in the presence of a selective PDE3 inhibitor (motapizone) was 100% efficacy on TNF- α release achieved.¹⁰ This additive increase of efficacy by combined PDE4 and PDE3 inhibition was demonstrated with rolipram and piclamilast and is in accordance with the small but significant PDE3 content of monocytes (Table 2). Whereas eosinophils and neutrophils contain exclusively PDE4 and are maximally inhibited by PDE4 inhibitors, in monocytes the small amount of PDE3 present in these cells (Table 2) obviously contributes to cAMP hydrolysis.

MACROPHAGES AND DENDRITIC CELLS

In monocyte-derived macrophages this situation was found to be even more pronounced since neither rolipram, piclamilast or motapizone reduced TNF- α release more than 20%. If a combination of PDE4 and PDE3 inhibitor was added complete inhibition could be achieved.¹⁰ This efficacy of the simultaneous

PDE4/3 inhibition was further supported by the similar efficacy of tolafermitine – a mixed-type PDE4/3 inhibitor. This complete inhibition of TNF- α release by PDE4/3 inhibition inclines to suggest that neither PDE1 nor PDE7 contribute significantly to endogenous cAMP hydrolysis under the given conditions. Interestingly, in macrophages efficacy of PDE inhibitors on TNF- α release was only observed in the additional presence of an adenylyl cyclase activator such as PGE₂, indicating a low level of basal adenylyl cyclase activity in these cells. Analogous synergy of PDE3 and PDE4 content is described for monocyte-derived dendritic cells.²¹

T LYMPHOCYTES, MAST CELLS AND ENDOTHELIAL CELLS

Activation of highly purified T lymphocytes leading to proliferation and cytokine release has been assessed in vitro by addition of various mitogens or other stimuli.²² Selective PDE4 inhibitors reduce proliferation or secretion of IL-2 following phytohemagglutinin challenge by about 40–60%.^{23–25} This efficacy was considerably enhanced if either a combination of PDE3 and PDE4 inhibitor or a mixed-type inhibitor such as zardaverine were used.^{12,24} These functional results are consistent with the presence of PDE3 and PDE4 in these cells.²⁶

Human mast cells contain equal amounts of PDE3 and PDE4 activity as measured by Tenor et al.¹ The corresponding influence of PDE inhibitors on release of histamine, PGD₂, and LTC₄ was recently described by Weston et al.²⁷ Neither piclamilast or rolipram nor siguazodan as mono-selective inhibitors had any functional influence at maximum selective concentrations. Although combinations of these drugs were not studied, IBMX as a non-selective PDE inhibitor reduced PGD₂ and LTC₄ release by >90% and

histamine release by about 50%. These results indicate that even if either PDE3 or PDE4 is completely inhibited the remaining cAMP-hydrolysing activity avoids increases of endogenous cAMP sufficient for protein kinase A activation. Simultaneous reduction of both activities PDE3 and PDE4 appears to be essential for attenuation of mast cell function.

For endothelial cells a similar lack of efficacy of mono-selective inhibition of PDE4 or PDE3 was described for protection vs. thrombin or H_2O_2 -induced increase of transendothelial permeability. In contrast, the endothelial layer was efficaciously protected against hyperpermeability only in the combined presence of PDE4 and PDE3 inhibitor or a mixed-type inhibitor such as zardaverine.^{11,23}

BRONCHIAL AND VASCULAR SMOOTH MUSCLE

In spontaneously contracted human bronchi mono-selective PDE3 inhibitors are potent relaxants on their own whilst PDE4 inhibition is ineffective.²⁴ Higher relaxing efficacy of PDE4 inhibitors was observed upon stimulation of bronchial constriction by histamine.^{24,25} However, combined inhibition of PDE4 and PDE3 triggers relaxation to a greater extent than PDE3 inhibitors alone, indicating a synergistic effect.²⁶ These results may indicate that, in contrast to inflammatory cells where PDE3 inhibition hardly affected functional effects in human bronchial smooth muscle cells, PDE3 is located in a compartment more closely associated to regulation of Ca^{2+} fluxes affecting contractility.

A slightly different picture was observed measuring the influence of PDE inhibitors on human smooth muscle proliferation in culture which had previously shown to be antagonized by protein kinase A activation.²⁷ In a recent study by Johnson-Mills et al.,²⁸ the analytical and functional data of cultures from four donors are presented separately. It occurs that in each donor the absolute amount of cAMP-hydrolysing activities varies by a factor of 2 while the ratios of PDE3/PDE4 activities are similar to approximately 2:1. Functionally, however, the mono-selective inhibitors rolipram or CI-930 alone have weak effects, whereas the combination of both compounds seems to inhibit proliferation in a supra-additive fashion. The predominance of PDE3 and PDE4 inhibition differs between donors and this functional difference does not meet the individual PDE3/PDE4 activity ratio, suggesting a transient intracellular regulation of PDE activity which is not detected by measuring activity in cell extracts.

Table 3 Adverse events of mono-selective PDE inhibitors

	PDE3-inhibition	PDE4-inhibition
Adverse events	Tachycardia Arrhythmia SVRI-reduction CI-increase Platelet inhibition	Nausea Vomiting Excess acid secretion Headache
Avoidance strategy	PDE3 A/B Selectivity?	PDE4 LAR Selectivity

ADVERSE EVENTS

Considering the PDE isoenzyme profiles and the corresponding functional influences of PDE inhibitors on those cells which are central to asthma pathology, it appears that mono-selective PDE4 inhibitors will affect several of these cells, in particular inflammatory cells. In the additional presence of a PDE3 inhibitor, however, the attenuation of cell activation will be more efficacious, at least in those cells containing both enzymes. The question arises of whether this higher efficacy of PDE4/3 inhibitors will be paralleled by a higher risk for side-effects.²⁹

Adverse events which have been observed in clinical studies with PDE3 and PDE4 inhibitors are listed in Table 3. Nausea and emesis were the most prominent adverse drug reactions associated with PDE4 inhibition, limiting their clinical use so far. From an intensive research for new PDE4 subtype-specific and conformation-selective compounds new drugs seem to have emerged which are characterized by higher tolerability. For the recombinant PDE4 splice variants PDE4A4 and PDE4D3 ('long forms' with >700 aa) two different binding affinities for rolipram with K_D values in the range of 1–5 nM and 0.1–1 μ M have been analysed,³¹ probably reflecting two different conformations of these enzymes.³ At artificial 'short forms', which resemble PDE4D2, however, predominantly a low affinity binding site ('low-affinity receptor', LAR) for rolipram was found.^{34,35} In functional experiments with monocytes,^{36,37} and T cells³⁸ (derived from the guinea-pig) and using various PDE4 inhibitors it was found that the low affinity binding site (LAR) for rolipram was involved in cAMP hydrolysis inhibition. High affinity binding, on the other hand, to the respective conformations of 'long-form' PDE4 splice variants was found to be related to ROS release from neutrophils,³⁹ and is supposed to be related to generation of emesis.^{40,41} Consequently, new compounds which, in contrast to rolipram, do not preferentially interact with the high affinity binding site such as piclamilast, should induce less emesis.⁴⁰ At optimum, compounds with preferential affinity for LAR (Table 3) should avoid generation of emesis while retaining antiinflammatory activity – although

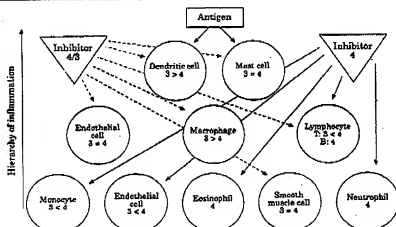


Fig. 3 Expected effects of PDE4 and PDE4/3 inhibitors in asthma derived from the PDE isoenzyme pattern of individual cell types.

targeting a limited number of cells involved in inflammation. The clinical studies with Arifo™ suggest that it may be possible to separate the potential for emesis and the antiinflammatory activity of PDE4 inhibitors.⁴¹

Mono-selective PDE3 inhibitors such as milrinone exhibited an improvement of myocardial performance in patients with cardiac failure. However, this benefit was associated with an increased risk for the occurrence of fatal arrhythmias, particularly in those patients with preexisting electrical instability of the myocardium. In fact, animal experiments have demonstrated that under conditions of ischemia/reperfusion excessive cAMP increases trigger arrhythmogenicity based on the induction of delayed afterdepolarizations. On the other hand, there is broad evidence suggesting that the arrhythmogenic potential of PDE3 inhibitors is limited to electrically unstable myocardium as manifested in ischemia/reperfusion events.^{42,43}

- Delayed afterdepolarizations (DAD) have been identified as important triggers of fatal arrhythmias. Ca^{2+} overload as observed in consequence of myocardial injury may provoke DAD by enhanced Na^+ influx based on Ca^{2+} -induced activation of the $\text{Na}^+/\text{Ca}^{2+}$ exchange channel. cAMP increases in the myocardial cell may aggravate a preexisting Ca^{2+} -overload by enhancing Ca^{2+} influx over the sarcolemmal membrane and indirect stimulation of Ca^{2+} -induced Ca^{2+} release from sarcoplasmic reticulum; thus increasing the risk of fatal DAD. On the other hand, based on this mechanism it appears evident that cAMP may have much less potential to trigger DAD in normal cardiomyocytes i.e. in the absence of Ca^{2+} -overload.
- Certainly, under the conditions of cardiac failure

there is a massive excess of circulating catecholamines. These catecholamines may amplify PDE3 inhibitor-induced cAMP increase and thus potentiate the arrhythmogenic risk. In contrast, in the absence of excess catecholamines as under physiological conditions the risk for PDE3 inhibitors to induce DAD and hence arrhythmias should be limited.⁴⁴

- Based on experiments in normal human atrial cardiomyocytes it may be hypothesized that PDE2 activated by cGMP formed by physiological levels of NO may partly cleave cAMP elevated by PDE4/3 inhibition, thus preventing excess cAMP formation. This may represent another mechanism indicating a low arrhythmogenic risk of PDE4/3 inhibition in the normal human heart. In contrast, in the injured myocardium high NO levels are formed that may potentiate cardiomyocyte Ca^{2+} overload and result in more complete cGMP-induced inhibition of PDE3.^{45,46}
- Any arrhythmogenic potential of PDE3 inhibition may only be revealed by the presence of Ca^{2+} -overload, excess circulating catecholamines and NO as this may happen in ischemia/reperfusion injury. In contrast, PDE3 inhibition does presumably not result in arrhythmias in electrically stable myocardium.

We advocate that in asthmatics with electrically stable myocardium an increased arrhythmogenic risk should not be inherent to PDE4/3 inhibitors.

Independent from this discussion another strategy for avoiding eventual cardiac side effects may be possible. Cardiac tissue contains predominantly PDE3A whilst PDE3B has been detected in inflammatory cells.⁴⁷ PDE4/3 inhibitors with PDE3B

selectivity thus might avoid any eventual cardiac risk (Table 3).

CONCLUSIONS

The inflammatory and effector cells present in the asthmatic airways are arranged in a putative hierarchy in Fig. 1. Regulating cells are in the top and medium levels whereas effector cells which depend on signals and interactions of the orchestrators are found in the bottom level. Contents and ratios of PDE3 and PDE4 activities are included as well as the functional influences of mono-selective PDE4 and dual-selective PDE4/3 inhibitors, as measured in vitro in human cells and tissue preparations.

It is evident that PDE4 inhibitors will strongly affect the effector cells and reduce (1) their mediator and enzyme release, (2) decelerate their chemotaxis and (3) indirectly relax bronchi by inhibition of release of bronchoconstricting mediators, thus normalizing several of the cellular abnormalities in asthma. The combined PDE4/3 inhibitors will exert higher efficacy compared to PDE4 inhibitors at orchestrating cells such as T lymphocytes, dendritic cells or mast cells. Further, PDE4/3 inhibitors trigger direct bronchodilation. Thus, protection against allergen-induced mast cell-mediated bronchoconstriction should be achieved efficiently both on the level of mast cell inhibition and direct bronchodilation. In addition, a retardation of remodelling-associated smooth muscle cell proliferation may be predicted.

Thus, from the in vitro experiments, a preponderance of PDE4/3 inhibition is evident. However in areas of inflammation iNOS is expressed and corresponding high NO levels will raise intracellular cGMP levels. Therefore, under inflammatory conditions an endogenous inhibition of PDE3 has to be expected which would not be evident under in vitro conditions. In vivo studies have to decide whether combined PDE4/3 inhibitors offer a real advantage over mono-selective PDE4 inhibitors.

For both PDE4 and PDE4/3 inhibitors a high potential as an antiasthma drug can be predicted, with PDE4/3 inhibitors having an even higher efficacy.

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Identification and Function of Cyclic Nucleotide Phosphodiesterase Isoenzymes in Airway Epithelial Cells

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Epithelial cells actively participate in inflammatory airway disease by liberating mediators such as arachidonate metabolites and cytokines. Inhibition of phosphodiesterases (PDEs) may be a useful anti-inflammatory approach. The PDE isoenzyme pattern and the effects of PDE inhibition on mediator generation were analyzed in primary cultures of human and porcine airway epithelial cells (AEC) and in the bronchial epithelial cell line BEAS-2B. PDE4 and PDE5 were detected in lysates of all cell types studied. In primary cultures of human AEC, the PDE4 variants PDE4A5, PDE4C1, PDE4D2, and PDE4D3 were identified by polymerase chain reaction analysis. Evidence of the recently described PDE7 was obtained by rolipram-insensitive cyclic adenosine monophosphate (cAMP) degradation, and its presence was verified by the demonstration of PDE7 messenger RNA. Primary cultures of human airway epithelium also expressed PDE1. Enhanced epithelial cAMP levels, induced by forskolin and PDE4 inhibition, increased formation of prostaglandin E_2 (PGE $_2$), but not of interleukin (IL)-8 or 15-hydroxyicosatetraenoic acid (15-HETE) in airway epithelial cells. Increased cyclic guanosine monophosphate levels in these cells provoked by sodium nitroprusside and the PDE5 inhibitor zaprinast reduced the PGE $_2$ synthesis, whereas 15-HETE and IL-8 formation were unchanged. The data suggest that PDE isoenzymes are important in airway inflammation and that PDE inhibitors exert anti-inflammatory effects by acting on AEC. Fuhrmann, M., H.-U. Jahn, J. Seybold, C. Neurohr, P. J. Barnes, S. Hippenstiel, H. J. Kraemer, and N. Suttorp. 1999. Identification and function of cyclic nucleotide phosphodiesterase isoenzymes in airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* 20:292-302.

In industrialized countries, inflammatory airway diseases are frequent disorders with increasing incidence. The pathogenesis of these diseases is complex and poorly understood. Recent studies, however, suggest that in the

broad array of cells involved in orchestrating airway inflammation (e.g., lymphocytes, mast cells, eosinophils, granulocytes), the airway epithelium itself plays a prominent and active role. Indeed, epithelial cell-derived mediators such as interleukin (IL)-8, 15-hydroxyicosatetraenoic acid (15-HETE), tumor necrosis factor- α (TNF- α), granulocyte macrophage colony-stimulating factor, nitric oxide (NO), and prostaglandin E_2 (PGE $_2$) promote or reduce airway inflammation. In addition, bronchial epithelial cells are also targets of a variety of mediators released by neighboring cells (NO, TNF- α , etc.) (1-4).

Currently available therapies for inflammatory and obstructive airway disease such as β_2 -adrenoceptor agonists or glucocorticoids are nonspecific and not without side effects. One group of reagents with powerful anti-inflammatory activity consists of inhibitors of cyclic nucleotide phosphodiesterases (PDE). Thus far, seven PDE gene families encoding multiple PDE proteins have been identified (5-7). The PDE classification is based on substrate specificity and regulatory characteristics. The PDE isoenzyme pattern differs among tissues and cells. Our recent analysis in endothelial cells showed high activities of PDE2, PDE3, and PDE4 (8-10). These cells, however,

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Abbreviations: 50 μ M arachidonic acid 10 μ M A23187, A23187; airway epithelial cells, AEC; basal pair(s), bpi; bovine pituitary extract, BPE; bovine serum albumin, BSA; cyclic adenosine monophosphate, cAMP; complementary DNA, cDNA; cyclic guanosine monophosphate, cGMP; erythro-9-(2-hydroxy-3-nonyl)adenine, EHNA; epidermal growth factor, EGF; enzyme-linked immunosorbent assay, ELISA; 15-hydroxyicosatetraenoic acid, 15-HETE; Hanks' balanced salt solution, HBSS; horseradish peroxidase, HRP; 3-isobutyl-1-methyl-xanthine, IBMX; interleukin, IL; messenger RNA, mRNA; octadecylsulfonyl, ODS; phosphate-buffered saline, PBS; phosphodiesterase, PDE; prostaglandin E_2 , PGE $_2$; radioimmunoassay, RIA; 4-(1'-cyclopentyl-1'-methoxyphenyl)-2-pyrrolidone, rolipram; reversed-phase high pressure liquid chromatography, RP-HPLC; reverse transcription-polymerase chain reaction, RT-PCR; sodium nitroprusside, SNP; tumor necrosis factor- α , TNF- α .

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lacked PDE5, the major cyclic guanosine monophosphate (cGMP)-degrading PDE. Airway epithelial cells (AEC) probably have an efficient cGMP-metabolizing capacity because they are continuously exposed to NO in the inhaled and exhaled air (11). The cyclic adenosine monophosphate (cAMP)-specific PDE4 has been demonstrated in many proinflammatory cell types, and PDE4 inhibition is a conceivable useful anti-inflammatory approach (12). Smooth-muscle cells possess PDE1-5; and inhibition of the cAMP-specific PDE5 regulates the tone of human peripheral airways (13).

The first objective of the present study, therefore, was to identify the PDE isoenzymes in AEC. This appears to be essential if a specific and site-directed therapy with both anti-inflammatory and antiostructutive properties is to be developed. Thus, we established AEC of porcine and human origin and also used the human bronchial epithelial cell line BEAS-2B.

The second objective of the present study was to test the concept that PDE inhibition alters the secretion of mediators and cytokines in AEC. It is well established that in the presence of enhanced intracellular levels of cyclic nucleotides, monocytes, T lymphocytes, eosinophils, and granulocytes demonstrate a decreased respiratory burst and cytokine generation (12, 14). Similarly, in endothelial cell monolayers, elevated cyclic nucleotides block endothelial hyperpermeability (8-10). Collectively, cAMP and cGMP appear to be important regulators of the inflammatory reaction. To test this concept, we focused on epithelial generation of IL-8 and of the lipid mediators PGE₂ and 15-HETE.

Materials and Methods

Materials

Tissue-culture plasticware was obtained from Becton-Dickinson, Heidelberg, Germany. Medium 199, serum-free keratinocyte medium, Dulbecco's modified Eagle's medium (DMEM)-F12, fetal calf serum (FCS), Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS), N/2-hydroxypropylazirine N'-ethanesulfonic acid (Hepes), epidermal growth factor (EGF), L-glutamine, and bovine pituitary extract (BPE) were from Gibco, Karlsruhe, Germany. Deoxyribonuclease type I (DNase), trypsin blue, and antibiotics were from Boehringer Mannheim GmbH, Mannheim, Germany. Anticollagenin (clone MNF 116) and avidin-biotin-horse radish peroxidase (HRP) were supplied from Dakopatts GmbH, Hamburg, Germany. Antivimentin (clone V9) was obtained from Dianova-Immunotech, Hamburg, Germany. Biotinylated horse antimouse antibody, Vectastain alkaline phosphatase kit, p-nitrophenylphosphate, and avidin-biotin blocking reagent were from Vector Laboratories, Inc., Burlingame, CA. The monoclonal antibody directed against PGE₂ was generously provided by K. Brune and J. Mollenhauer, Institute of Pharmacology, University of Erlangen, Germany. Recombinant human TNF- α (2×10^7 U/mg) was obtained from R&D Systems, Wiesbaden, Germany. Gelatin from porcine skin type I, protease XIV, insulin, bovine apotransferrin, forskolin, 3-isobutyl-1-methyl-xanthine (IBMX), Crostafus arow snake venom, dipyrdimole, sodium nitroprusside (SNP), bovine

serum albumin (BSA), methyl formate, soybean trypsin inhibitor (SBTI), leupeptin, pepstatin, benzamide, dithiothreitol (DTT), calmodulin, and W7 were purchased from Sigma Chemical Co., Munich, Germany. Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) was provided by Dr. Podzurweit, Max Planck Institute of Experimental Cardiology, Bad Nauheim, Germany. 4-(3'-cyclopentyl-4'-methoxyphenyl)-2-pyrrolidone (rolipram) was kindly provided by Schering AG, Berlin, Germany; and 4, 5-dihydro-6-(4-(1H-imidazol-1-yl)-2-thienyl)-5-methyl-3-(2H)-pyridazinone (motaipzone) was from Rhone-Poulenc Rorer GmbH, Cologne, Germany. Enoximone was supplied by Dr. H. D. Görlach, Marion Merrell Dow Pharmaceuticals, Berlin, Germany. 6-(difluoromethoxy-3-methoxyphenyl)-3-(2H)-pyridazinone (zardaverine) was provided by Dr. Schudt, Byk Gulden GmbH, Konstanz, Germany. Vilmopredine, 8-methoxy-methyl-1BMX, trifluoperazine, and zaprinast were obtained from Calbiochem, Bad Soden, Germany. Tritiated cAMP and cGMP, IL-8 enzyme-linked immunosorbent assay (ELISA), and the ¹²⁵I-cAMP and ¹²⁵I-cGMP assay systems were from Amersham Buchler, Braunschweig, Germany. The 15-HETE assay system was purchased from Paezel and Lorei GmbH, Frankfurt, Germany; and C₁₈ columns (100-mg bed size) were from JCT, Bad Homburg, Germany. Retinoic acid was provided by Biofluids Inc., Rockville, MD. QAE Sephadex A-25 columns were from Bio-Rad, Richmond, CA. All other chemicals used were of analytical grade.

Isolation and Culture of Porcine AEC

Tracheae and central bronchi were obtained from freshly slaughtered pigs and kept on ice during transportation. Cells were isolated according to Wu and Smith, with minor modifications (15). Specimens were rinsed with ice-cold HBSS, filled with protease solution (M199 containing 0.1% protease XIV, 0.001% DNase, 200 U/ml penicillin, 0.2 mg/ml streptomycin, and 10 μ g/ml amphotericin B) and incubated for 16 h at 4°C. The protease solution was removed, and the cells were harvested by gentle agitation, washed twice in serum containing medium, and resuspended in DMEM/F12 supplemented with 1% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 5 μ g/ml amphotericin B, 0.1 mg/ml L-glutamine, 5 μ g/ml BPE, 5 ng/ml EGF, 5 μ g/ml transferrin, 5 μ g/ml insulin, 0.1 μ g/ml retinoic acid, and 20 ng/ml T3. The number of isolated cells was determined in a Neubauer chamber, and viability was assessed by trypan blue (0.4%) exclusion. About 10^6 viable cells/cm² were plated on collagen (10 μ g/ml)-coated culture dishes, well plates, or chamber slides. The AEC were grown in a 5% CO₂ atmosphere at 37°C and reached confluence in 7 to 10 d. Confluent primary cultures of porcine AEC were then used.

Isolation and Culture of Human AEC

Human tracheae and central bronchi were obtained from the Department of Pathology, University of Giessen. Preparations originated from patients who had died of a nonpulmonary disease and who had not been intubated. Human AEC were isolated according to the protocol described above, with the exception that exposure to protease solu-

tion was extended to 20 h. Only primary cultures of confluent AEC were used for the studies.

Culture of BEAS-2B Cells

A generous gift of Dr. Curtis Harris, National Institutes of Health, Bethesda, MD (18). BEAS-2B cells were grown on a matrix composed of 50 ng/ml fibronectin, 50 ng/ml vitronectin, and 0.01% BSA (fatty acid-free) in serum-free keratinocyte medium containing 0.1 µg/ml retinoic acid, 5 µg/ml BPE, 5 ng/ml EGF, 0.5 µg/ml epinephrine, 0.1 mg/ml L-glutamine, 100 U/ml penicillin, 0.1 µg/ml streptomycin, and 5 µg/ml amphotericin B. Cells were incubated in a 5% CO₂ atmosphere at 37°C and reached confluence within 14 d. For cell passage, monolayers in T75 flasks were exposed to trypsin-ethylenediaminetetraacetic acid solution (80 µg/ml trypsin) for 5 to 10 min at 37°C. After addition of 8 ml serum-free keratinocyte medium, the cell suspension was centrifuged at 800 rpm at 4°C for 5 min. Medium was removed and cell pellets were incubated in 200 µl SBT solution (30 mg/ml) for 2 min, resuspended in complete medium, and passaged with a 1:3 split.

549 cells were cultured according to Kwon and colleagues (17).

Characterization of Isolated and Cultured AEC by Immunohistochemistry

Isolated epithelial cells were characterized by morphologic and immunocytochemical criteria. AEC were grown on chamber slides and used for immunohistochemical examination after 8 d in culture. Cells were washed, permeabilized, and fixed with cold (-20°C) acetone/methanol (1:1). Preparations were then saturated with avidin-biotin blocking reagent and with PBS containing 0.1% horse serum to avoid nonspecific binding. Thereafter, slides were incubated with mouse anticytokeratin or mouse antivimentin antibody. After careful removal of excess primary antibodies, a biotinylated horse antirabbit antibody was added, followed by alkaline phosphatase ABC solution. After visualization with *p*-nitrophenylphosphate (2 mg/ml), slides were washed extensively, embedded, and photographed.

Assay of PDE

To determine PDE activity in AEC, cyclic nucleotide hydrolyzing activities were determined in cell lysates as described previously (8). Medium of T75 flasks was removed and cells were scraped in HBSS and washed twice by centrifugation. Cell pellets were resuspended in 200 µl ice-cold homogenization buffer (PBS containing 10 mM HEPES, 1 mM ethyleneglycol-*bis*-(β -aminoethyl) ether)-N,N'-tetraacetic acid, 1 mM MgCl₂, 5 mM DTT, 5 µM pepstatin, 10 µM leupeptin, 10 µM trypsin inhibitor, and 2 mM benzamide) and disrupted by sonification (ten pulses, 1 s each, on ice at a power output of 60 W). Protein concentration was measured using a commercially available protein reagent (Bio-Rad, Munich, Germany), using BSA as the standard. Cell lysates were kept on ice and PDE assays were performed within 5 min. PDE activity was determined as previously described (8, 9). A standard reaction mixture containing 60 mM Tris (pH 7.4), 5 mM MgCl₂, 1.25 mM CaCl₂, 100 µM calmodulin, and 0.5 µM cyclic nucleotide-³H-labeled

cyclic nucleotide (about 30,000 counts per minute) was used in a total volume of 200 µl. The reaction was initiated by addition of cell lysates (40 to 50 µg protein) and carried out at 37°C for 15 min. The reaction was stopped by adding 50 µl 0.2 N HCl and immediate cooling on ice for 10 min. Following incubation with 5'-nucleotidase (*C. arax* snake venom, 50 µl, 2 mg/ml in 400 mM Tris, pH 8.5) at 37°C for 15 min, 200-µl aliquots of the assay volume were loaded on QAE-Sephadex A-25 columns (1-ml bed volume). The columns were eluted with 2 ml 30 mM ammonium formate (pH 6.0) directly into scintillation vials. Results were corrected for blanks using denatured protein.

Determination of PDE Isoenzyme Activities

With exception of PDE7, PDE isoenzyme activities in AEC lysates were determined by quantifying the inhibition of PDE activity in the presence of 1 µM motapipone (PDE3), 10 µM rolipram (PDE4), 10 µM zaprinast (PDE5), 100 µM EHNA (PDE2), or 100 µM vinpocetine or 8-methoxymethyl-IBMX (PDE1). Overall PDE activity was blocked by 100 µM IBMX. The presence of PDE7 was suggested by demonstration of messenger RNA (mRNA) encoding for this PDE isoenzyme (see REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION section, below, for details). Because of the lack of a specific PDE7 inhibitor, the activity of this isoenzyme was calculated as the difference between PDE4-related and IBMX-insensitive cAMP-hydrolysis (see Table 1).

Determination of Cellular Cyclic Nucleotides

Cellular cyclic nucleotide content was measured by radioimmunoassays (RIAs) using commercially available [¹²⁵I]-assay systems as described (8, 9, 18, 19). Confluent epithelial cells in 24-well plates (about 600,000 cells/well) were incubated with specific PDE inhibitors in HBSS 15 min before and throughout the experimental period. Cells were then extracted twice with 500 µl ice-cold 65% ethanol. Extracts were pooled, evaporated under a stream of nitrogen by 56°C, and dissolved in assay buffer. Aliquots of the extracts and of the standards were acetylated by addition of acetic anhydride and triethylamine (1:2) to enhance the sensitivity of cyclic nucleotide detection.

Determination of 15-HETE by Reversed-Phase High Pressure Liquid Chromatography (RP-HPLC) and ³H-RIA

Eicosanoids were extracted from cell supernatants by octadecylsilyl (ODS) solid-phase extraction columns (Bond Elute LRC solid phase C₁₈ cartridges, 100 mg sorbent). Cartridges were conditioned with 10 ml 100% methanol, 10 ml methanol/water/acetic acid (72.6/27.3/0.1 vol/vol/vol, pH 5.1), and 10 ml distilled water. Samples were then applied to the cartridges, washed with 10 ml distilled water, and eluted with 600 µl of methanol, followed by 2 × 600 µl of formic acid methyl ester. Eluates were vacuum-evaporated and dissolved in 30 µl 100% methanol. The RP-HPLC was carried out on ODS columns (250 × 2 mm; Hypersil ODS 3-µm particles) at a constant flow of 200 µl/min (Gynkorek pump, model 480) using methanol/water/acetic acid (72.6/27.3/0.1 vol/vol/vol, pH 5.1) as mobile phase. Online detection and quantification of the eluted

TABLE 1
Cyclic nucleotide hydrolysis* by AEC

	Purified AEC		BEAS-2B		Human AEC	
	cAMP	cGMP	cAMP	cGMP	cAMP	cGMP
Total hydrolysis	1.81 ± 0.16	3.04 ± 0.57	9.31 ± 0.60	6.78 ± 0.90	0.90 ± 0.04	0.58 ± 0.04
Controls ¹	0.51 ± 0.08	1.11 ± 0.20	0.91 ± 0.03	1.00 ± 0.14	0.25 ± 0.09	0.00 ± 0.03
Rolipram, 10 μ M ²	0.77 ± 0.17	(-)	2.62 ± 0.53	(-)	0.58 ± 0.03	(-)
Zaprinast, 10 μ M ³	(-)	1.03 ± 0.14	(-)	1.09 ± 0.05	(-)	0.10 ± 0.06
PDE7-related**	0.23 ± 0.11	(-)	1.78 ± 0.49	(-)	0.32 ± 0.08	(-)
Total hydrolysis ^{1†}	(-)	(-)	(-)	(-)	(-)	0.62 ± 0.12
Vinpocetine, 100 μ M ^{4‡}	(-)	(-)	(-)	(-)	(-)	0.41 ± 0.04

The cyclic nucleotide hydrolysis in lysates of AEC in the absence and presence of PDE isoenzyme-specific inhibitors is shown. Inhibition of PDE2 and PDE3 was without effect on cyclic nucleotide hydrolysis in all cell types studied (data not shown).

* nmol \times min⁻¹ \times mg protein⁻¹.

¹ Controls indicate PDE-independent cyclic nucleotide hydrolysis in the presence of 100 μ M IBMX.

² Inhibition of PDE4 by rolipram resulted in decreased hydrolysis in all cell types studied.

³ Inhibition of PDE5 by zaprinast resulted in decreased hydrolysis in all cell types studied.

⁴ (-) indicates that values are not significantly different from total cyclic nucleotide hydrolysis.

** PDE7-related activity is calculated as the difference in cAMP hydrolysis between IBMX- and rolipram-treated samples.

[†] Total cyclic nucleotide hydrolysis in human AEC in the presence of 1.25 mM CaCl₂ and 100 μ M calmodulin.

[‡] Inhibition of PDE1 activity (in the presence of 1.25 mM CaCl₂ and 100 μ M calmodulin) by vinpocetine reduced cGMP hydrolysis.

compounds was carried out using a UVIS VARIO 2 photometer/Spectra Physics integrator at 237 nm, with commercially available 15-HETE as standard reagent. The recovery determined under the conditions outlined above using different quantities of 15-HETE was 72 ± 3% ($n = 10$). Additionally, 15-HETE synthesis was measured using a commercially available ³H-RIA system. Supernatants of stimulated and control AEC were extracted as described for the RP-HPLC procedure. Samples were resolved in RIA buffer and assayed according to the manufacturer's instructions. All 15-HETE data obtained were corrected for their respective recovery of the overall analytical procedure and are expressed as nanograms of 15-HETE per 10⁶ AEC.

Determination of PGE₂

PGE₂ in AEC supernatants was examined using an avidin-biotin-HRP-based ELISA system as described (20). Conjugates of PGE₂ with BSA were prepared by carbodi-

imide coupling and were then separated from uncoupled prostanoic acid and free BSA by gel filtration on a Pharmacia G20 column. ELISA plates were coated with 200 μ l of diluted PGE₂ conjugate solution (1 μ l/ml in 46 mM Na₂CO₃/NaHCO₃, pH 9.6) and incubated overnight at 4°C. After washing, a 100- μ l sample volume was applied to each well, followed by 100 μ l anti-PGE₂-antibody solution (5 μ g/ml in PBS containing 2% BSA) for 16 h. ELISA was completed by adding biotinylated antimuscle antibodies and biotin-HRP as described (20). Results are expressed as nanograms of PGE₂ per 10⁶ AEC.

IL-8 ELISA

IL-8 determination was performed using a commercially available IL-8 ELISA system (Amersham). Aliquots (100 μ l) of supernatant of resting and TNF- α -stimulated cells were applied to a 96-well plate, and the ELISA was performed according to the manufacturer's instructions. Results are expressed as nanograms of IL-8 per 10⁶ AEC.

TABLE 2
Primers and conditions used in RT-PCR experiments

Gene Product	Deoxyoligonucleotide Sequences	Product Size (bp)	Annealing Temperature (°C for 60 s)	Cycles	References
PDE4A5	Forward 5'-ACC AAT GTG CCC GTT CCC AGT-3' Reverse 5'-GCC TCC AGC GTA ATC CGA CA-3'	570	65°	32	13
PDE4B2	Forward 5'-AGG GCA GCC TGA GGT ATT AAA-3' Reverse 5'-CAC TCC TGG GTT ACA GTT GTA-3'	364	62°	33	13
PDE4C1	Forward 5'-GGA GTT GGC TGA CAC TGA ACT-3' Reverse 5'-AGA AAG ACA CCA CGG CAT CGT-3'	336	68°	32	13
PDE4D1	Forward 5'-TAT GCG AGG ATC CCC CCC TT-3' Reverse 5'-CGG TTA CCA CAC AAC TCT CTC-3'	530	65°	34	13
PDE4D2	Forward 5'-AGC ATG GCG GGA GGA GCG CTA-3' Reverse 5'-CGG TTA CCA CAC AAC TCT GTC-3'	456	68°	32	Unpublished data
PDE4D3	Forward 5'-GCA AGA TCG AGC ACC TAG CAA-3' Reverse 5'-CGG TTA CCA CAC AAC TCT GTC-3'	516	68°	30	Unpublished data
PDE7A1	Forward 5'-CGA GGT GGT ATT TAA GCA AGC-3' Reverse 5'-TCC TCA CTG CTC GAC TGT TCT-3'	260	59°	23	13



Figure 2. Expression of PDE7 mRNA in human AEC. An ethidium bromide-stained agarose gel of the RT-PCR products derived from human mRNA is shown. In the presence of reverse transcriptase, both the extracted RNA of human AEC (lane 1) and human lung (lane 4, positive control) show a single band with the appropriate size (285 bp). In the absence of reverse transcriptase (lane 2) or RNA (lane 3), no RT-PCR products were synthesized.

established SV-40/adenovirus-transformed human bronchial epithelial cell line BEAS-2B, we studied confluent primary cultures of AEC of porcine and human origin. The purity of the cells used was > 97%, as indicated by morphologic and immunocytochemical criteria. These cells were cytokeratin-positive and vimentin-negative. Porcine cultured pulmonary artery endothelial cells (8, 9, 18, 23) were used as vimentin-positive controls. The major cyclooxygenase and lipoxigenase products in porcine AEC studied were PGE₂ and 15-HETE (24).

PDE Isoenzyme Activities in AEC

All three cell types studied hydrolyzed both cyclic nucleotides with different efficiencies (Table 1). BEAS-2B displayed the highest total cAMP- and cGMP-metabolizing capacities, followed by porcine and human AEC. Allosteric regulators, specific inhibitors, and mRNA analysis were applied to study the PDE isoenzyme pattern in lysates of AEC. cAMP hydrolysis was reduced by 50 to 80% in the presence of the specific PDE4-inhibitor rolipram, whereas other specific inhibitors had no effect (Table 1, Figure 1), suggesting that PDE4 is the major cAMP-degrading en-

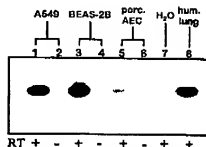


Figure 3. Expression of PDE7 mRNA in airway epithelial cells. Shown is the Southern analysis of cDNA of A549 cells derived by RT-PCR, BEAS-2B, porcine AEC (one of four samples analyzed), and human lung (lane 8, positive control). The cDNA fragments of all cell types studied hybridized with the cloned and sequenced cDNA for human PDE7 (lanes 1, 3, and 5). In the absence of reverse transcriptase (RT, lanes 2, 4, and 6) or RNA (lane 7) no PCR products were synthesized, indicating specific amplification of the 285-bp fragment corresponding to PDE7.

zyme in AEC. The remaining cAMP hydrolysis might be attributable to PDE7, which by definition is a rolipram-insensitive, cAMP-hydrolyzing enzyme (6, 7). Selective inhibitors for PDE7 are unknown and no specific antibodies were available to us to confirm this notion. We therefore evaluated the presence of mRNA for PDE7 expressed in AEC by RT-PCR using primers designed to recognize a

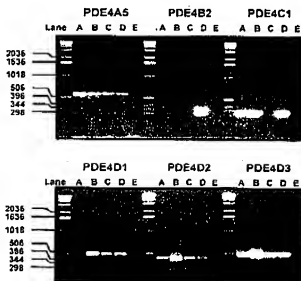


Figure 4. Expression of PDE4 mRNA of AEC. An ethidium bromide-stained agarose gel of the RT-PCR products derived from mRNA of primary cultured human AEC (lane A), A549 cells (lane B), BEAS-2B cells (lane C), and human lung (lane D) is shown. Human AEC expressed PDE4 variants 4A5, 4C1, 4D2, and 4D3. In the absence of reverse transcriptase (lane E), no RT-PCR products were synthesized. Human lung tissue expressed all PDE4 isoenzymes studied (lane D).

TABLE 4
Homology of the cloned and sequenced RT-PCR products to the published sequence of the PDE7 gene

Cell Type	Grade of Homology (%)	
	cDNA Sequence	Amino Acid Sequence
BEAS-2B	100	100
Human AEC	100	100
Porcine AEC	93.4	97.5

The grade of homology of the cloned RT-PCR products derived from BEAS-2B and human and porcine AEC to the published sequence of the PDE7 gene is shown. Identity with the human sequence (6) was shown for the fragments derived from BEAS-2B cells and human AEC. Protein shares a high homology compared with the human cDNA sequence. The amino acid sequence was evaluated from the cDNA.

unique sequence in the human gene (Tables 2 and 3). Figure 2 shows an ethidium bromide-stained agarose gel of a representative experiment demonstrating amplified cDNA fragments derived from human AEC corresponding to the predicted size of human PDE7 (285 base pairs [bp], Table 3). This was subsequently confirmed by cloning of the PCR products of all three airway epithelial cell types studied into pGEM-T-Vector, followed by double-stranded sequencing (100 and 93.4% homology for human and porcine AEC, respectively; Table 4). Southern blot analysis of the PCR products derived from BEAS-2B and porcine AEC with the human PDE7-cDNA probe also confirmed the presence of PDE7 in these cells (Figure 3). In these studies, RT-PCR products of mRNA extracted from A549 and human lung tissue were used as positive controls (Figure 3).

PCR analysis of human AEC indicated the expression of mRNA encoding for the PDE4 variants 4A5, 4C1, 4D2, and 4D3 (Figure 4). mRNA extracted from human lung tissue, A549 cells, and BEAS-2B cells served as controls. Primers used for amplification of PDE4 cDNA fragments and their corresponding PCR products were cloned and sequenced as previously described (14).

In the presence of the specific PDE5-inhibitor zaprinast, cGMP hydrolysis was inhibited by 80 to 100% (Table 1, Figure 1) whereas inhibitors of PDE2 and PDE3 (EHNA and moxipizone) had no effect (Figure 1), suggesting that PDE5 is the major cGMP-degrading enzyme in AEC.

The addition of vinpocetine or 8-methoxy-methyl-IBMX to lysates of BEAS-2B and porcine AEC (in the presence of calmodulin and Ca^{2+}) did not inhibit cGMP hydrolysis, indicating that PDE1 does not play a dominant role in cyclic nucleotide degradation in these cells (Table 1, Figure 1). In primary cultures of human AEC, however, vinpocetine was effective and reduced cGMP hydrolysis by approximately 20%, thereby suggesting the presence of a PDE1 (Table 1, Figure 1).

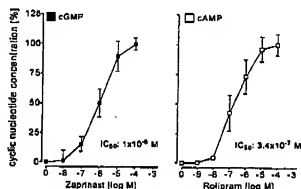


Figure 5. Concentration-dependent increase of cyclic nucleotides in porcine AEC. The accumulation of cAMP (open squares, right) and cGMP (filled squares, left) in the presence of increasing concentrations of zaprinast and rolipram is shown. Both inhibitors concentration-dependently increased cGMP or cAMP with IC_{50} values of 1 or 0.34 μM , respectively. Data are given as means \pm SEM of four to seven independent experiments.

Cyclic Nucleotide Content in Intact AEC

PDE isoenzyme activities in cell lysates were correlated with cyclic nucleotide levels in intact cells. Increasing concentrations of rolipram induced a concentration-dependent accumulation of cAMP in intact porcine AEC stimulated with 1 μM forskolin with an IC_{50} of 0.34 μM (Figure 5). Similarly, zaprinast induced a concentration-dependent accumulation of intracellular cGMP in intact AEC stimulated with SNP ($\text{IC}_{50} = 1 \mu\text{M}$) (Figure 5). Both inhibitors were used at 10 μM for subsequent studies.

Forskolin-stimulated porcine AEC and BEAS-2B cells showed a substantial cAMP accumulation in the presence of rolipram. Suppression of all other PDE isoenzymes did not enhance cAMP concentration, indicating that PDE4 is

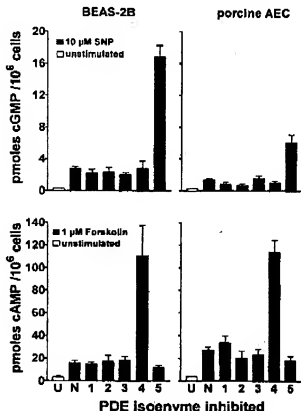


Figure 6. Cyclic nucleotide content in BEAS-2B and porcine AEC. The stimulated increases of cGMP (10 μM SNP) and cAMP (1 μM forskolin) in intact cells in the presence or absence (N) of PDE isoenzyme-specific inhibitors are shown. In BEAS-2B (top left) and porcine AEC (top right), cGMP levels increased significantly in the presence of the PDE5 inhibitor zaprinast only, whereas inhibition of all other PDE isoenzymes was without effect. As to cAMP, only inhibition of PDE4 with rolipram resulted in a significant accumulation of this cyclic nucleotide in BEAS-2B (bottom left) and porcine AEC (bottom right). Basal levels of cAMP and cGMP are shown as unfilled bars (U). Data represent means \pm SEM of eight to 11 independent experiments.

the major cAMP-degrading enzyme in AEC (Figure 6). Because of the lack of specific inhibitors, it is currently not possible to assess the contributions made by PDE7 to overall cAMP metabolism in intact cells.

With respect to cGMP hydrolysis, BEAS-2B and porcine AEC were stimulated with SNP. cGMP concentrations increased in presence of zaprinast (Figure 6) only, indicating the predominant role of PDE5 for cGMP metabolism. Data for human AEC were very similar, with the exception that cGMP also increased in the presence of a PDE1 inhibitor (Figure 7). Overall, these cyclic nucleotide results in intact AEC are consistent with the PDE isoenzyme pattern determined in cell lysates.

Effect of Cyclic Nucleotide Levels on the Generation of PGE_2 , 15-HETE, and IL-8 in AEC

To correlate AEC function with cyclic nucleotide levels, we focused on the ability of epithelial cells to generate PGE_2 , 15-HETE, and IL-8 (Figures 8 and 9). A 7-fold increase in PGE_2 formation was noted after exposure of porcine AEC to 50 μM arachidonic acid/10 μM A23187 (AA/A23) for 30 min (Figure 8, *bottom*). When cAMP was elevated first in AEC with forskolin/rolipram, there was a 4-fold increase in the effectiveness of the stimulus, resulting in an overall 28-fold increase in PGE_2 synthesis above baseline. Interestingly, preincubation of AEC with SNP/zaprinast to increase maximally cGMP content reduced the effectiveness of the stimulus for PGE_2 production by 40% (Figure 8, *bottom*). Forskolin and rolipram are highly specific reagents; on the other hand, SNP and zaprinast in high concentrations may have effects unrelated to increased cGMP levels.

15-HETE formation in porcine AEC was increased 7-fold within 10 min by AA/A23 (Figure 8, *top*). Maneuvers taken to elevate cyclic nucleotide levels did not modify the

stimulated synthesis of this lipooxygenase product. In control experiments we verified that the stimuli themselves (AA/A23) did not increase cyclic nucleotides in epithelial cells (data not shown).

Stimulation of BEAS-2B cells with 10 ng/ml TNF- α for 8 h increased IL-8 synthesis 9-fold (Figure 9). When cAMP or cGMP levels were increased first, TNF- α -induced IL-8 formation remained unchanged, suggesting that under the experimental conditions used, this epithelial cell mediator is not regulated by increased cyclic nucleotides.

Discussion

Three bronchial epithelial cell types were used for the identification of the PDE enzyme pattern. Besides the well-established human bronchial epithelial cell line BEAS-2B, emphasis was placed on the analysis of confluent primary cultures of AEC derived from human and porcine tissue (15). Morphologic and immunocytochemical criteria were applied to identify cells and to verify purity of the cultures. All epithelial cells were cytokeratin-positive and vimentin-negative; in the same set cultured pulmonary artery endothelial cells were used as vimentin-positive and cytokeratin-negative controls. The major cyclooxygenase and lipooxygenase products of epithelial cells were PGE_2 and 15-HETE; this arachidonic acid metabolite profile is similar to that described for human, bovine, and canine bronchial epithelial cells (3, 24–27).

Using a combination of biochemical, pharmacologic, and molecular techniques, we have demonstrated that primary cultures of human AEC express PDE1, PDE4, PDE5, and PDE7. We confirmed these observations also for primary cultures of porcine AEC and for the cell line BEAS-2B, both of which contained PDE isoenzymes 4, 5, and 7. Interestingly, these cells did not express PDE1,

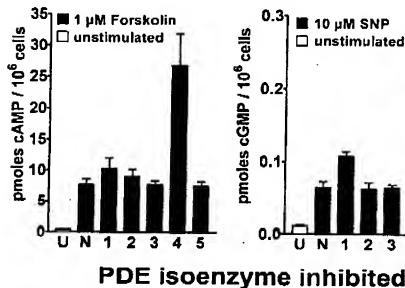


Figure 7. Cyclic nucleotide content in human AEC. Stimulated increases of cAMP (1 μM forskolin) and cGMP (10 μM SNP) in intact cells in the presence or absence (N) of PDE isoenzyme-specific inhibitors are shown. cAMP levels (left) increased significantly in the presence of rolipram. Inhibition of PDE5 (zaprinast) and PDE1 (vincetoxin) or 8-methoxymethyl-IBMX resulted in a significant accumulation of cAMP (right). Basal levels of cAMP and cGMP are shown as unstimulated bars (U). Data represent means \pm SEM of three independent experiments.

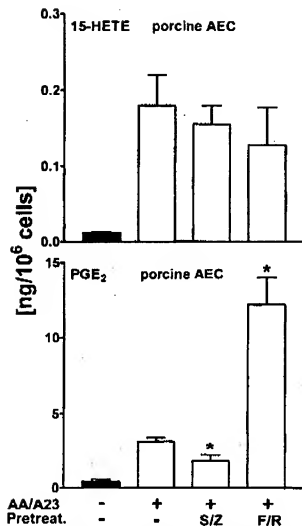


Figure 8. Effect of elevated cyclic nucleotides on AA/A23-induced generation of PGE₂ and 15-HETE in porcine AEC. Stimulation of 15-HETE and PGE₂ generation of porcine AEC was accomplished by exposure of cells to AA/A23 for 30 min. Two experimental sets of cells were pretreated with reagents prior to stimulation to increase cyclic nucleotides. cAMP was elevated by exposure of AEC to 1 μ M forskolin/10 μ M rolipram (F/R), and cGMP by exposure to 10 μ M SNP/10 μ M zaprinast (S/Z) for 10 min. Data indicate that AA/A23-stimulated PGE₂ synthesis was significantly enhanced in AEC with elevated cAMP levels and reduced in AEC with elevated cGMP levels. Stimulated 15-HETE formation in AEC was not influenced by enhanced cyclic nucleotides. Data appear as means \pm SEM of four to seven independent determinations.

which may be due to species differences or to special circumstances introduced by virus transformation. Similar data for primary cultures of human AEC and for the alveolar epithelial cell line A549 were reported in abstract form by Rabe and colleagues (28) and Robichaud and as-

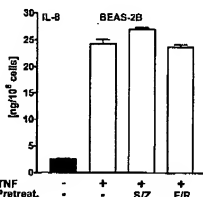


Figure 9. Effect of elevated cyclic nucleotides on TNF-induced generation of IL-8 in BEAS-2B cells. IL-8 synthesis was stimulated in BEAS-2B cells by 10 ng/ml TNF- α for 8 h. Two experimental sets of cells were pretreated with reagents prior to stimulation to increase cyclic nucleotides. cAMP was elevated by exposure of AEC to 1 μ M forskolin/10 μ M rolipram (F/R), and cGMP by exposure to 10 μ M SNP/10 μ M zaprinast (S/Z) for 10 min. Data indicate that TNF-stimulated IL-8 synthesis remained unchanged even in the presence of enhanced cyclic nucleotide levels in AEC. Data appear as means \pm SEM of four independent determinations.

sociates (29), although no information was provided with respect to PDE7 and PDE4 variants. We applied RT-PCR using primers specific for the four PDE4 genes and detected PCR products that correspond to PDE4A5, 4C1, 4D1, 4D2, and 4D3. Rousseau and coworkers (30) used biochemical and pharmacologic methods to identify PDE isoenzymes in bovine tracheal epithelial cells. In that study only one broad peak of activity was resolved by diethylaminoethyl anion exchange chromatography; that peak was characterized using isoenzyme-selective inhibitors and allosteric effectors. The authors reported high activities of PDE1 in their study (30). As to PDE1, species differences appear to prevail because this isoenzyme is present in human and bovine but not in porcine AEC.

The absence of PDE3 in AEC contrasts with data published by Kelley and associates, who described the functional effects of PDE3 inhibitors on chloride fluxes in Calu-3, 16HBE, and transformed nasal polyp cells (31, 32). However, in that study no direct evidence for PDE3 was provided. Because these authors focused on specialized epithelial cell mutants with different degrees of cystic fibrosis transmembrane conductance regulator activities, their data with respect to PDE3 probably cannot be generalized. Using *in situ* hybridization, Reinhardt and coworkers (33) studied PDE3B expression during embryonic and postnatal development. Although PDE3B mRNA was demonstrated in rat bronchial epithelial cells, this enzyme was not documented at the protein level. Thus, like PDE1, species differences also appear to exist for PDE3.

Considering all this data together, it appears that in AEC PDE4 (4A5, 4C1, 4D1, 4D2, and 4D3) is the major cAMP-degrading pathway, whereas PDE5 is primarily re-

sponsible for the metabolism of cGMP. Regarding primary cultures of human AEC, PDE1 accounts for at least 20% of the total cGMP hydrolysis, and PDE5 the remainder.

In addition to the large amount of rolipram-sensitive cAMP hydrolysis (PDE4), there was also consistently a substantial fraction of rolipram-insensitive cAMP degradation (25 to 40% of total cAMP hydrolysis), suggesting the presence of a PDE7 which, by definition, is a high-affinity, cAMP-specific, rolipram-insensitive PDE (5-7). Because of the lack of selective inhibitors, it is not possible to attribute unequivocally the rolipram-resistant cAMP-PDE activity to PDE7. Moreover, PDE7 activity will probably be underestimated because IBMX does not block all PDE7 activity (34) and because high concentrations of rolipram can—despite definition—reduce PDE7 by about 20% (6). Compelling evidence, however, to support the expression of PDE7 was provided by the unambiguous identification of PDE7 mRNA in AEC. To our knowledge this is the first demonstration of PDE7 in AEC.

Homogenates derived from human airways displayed activities for PDE1 to PDE5, with highest activities for PDE4 and PDE5, whereas PDE7 was not analyzed (13, 35). Previous studies using human airway smooth-muscle strips also revealed the presence of all PDE isoenzymes (36). The fact that AEC lack PDE2 and PDE3 suggests that airway smooth-muscle cells substantially contribute to the PDE isoenzyme pattern of airway homogenates. However, additional studies are required for an exact mapping of PDE isoenzymes in specific cell types of the airways.

On the basis of the PDE isoenzyme pattern analyzed, we tried to correlate AEC function with cyclic nucleotide levels and focused on the generation of PGE₂, 15-HETE, and IL-8. AA/A23 increased PGE₂ synthesis 7-fold in AEC. When cAMP was elevated first in AEC with forskolin/rolipram, there was a 4-fold increase in the effectiveness of the stimulus resulting in an overall 28-fold increase in PGE₂ synthesis above baseline and suggesting that PDE4 inhibition may enhance the anti-inflammatory and antiobstructive effects of endogenous PGE₂. Interestingly, increased cAMP levels reduced the effectiveness of the stimulus for PGE₂ production. Interpretation of these data in terms of airway physiology is complex. One possible explanation relates to the prevention of an unopposed bronchodilation by simultaneous action of cGMP- and cAMP-dependent mechanisms. If increased cAMP levels decrease PGE₂ with a subsequent decrease in cAMP, this mechanism will substitute for a cGMP-stimulated, cAMP-degrading activity (37). This imitation of a PDE2-like activity would allow crosstalk between the two cyclic nucleotide species and compensate for the absence of a PDE2 in AEC.

Next we studied the interrelationship between cyclic nucleotides and the major epithelial cell lipoxigenase product 15-HETE. The AA/A23-related synthesis of 15-HETE was unaffected in SNP/zaprinast- or forskolin/rolipram-pretreated AEC, suggesting that increased nucleotides act on the cyclooxygenase but not on the lipoxigenase pathway in AEC.

IL-8 is an important proinflammatory cytokine that is released from AEC during airway inflammation upon exposure to TNF- α , IL-1 β , and neutrophil elastase (4, 17, 38-40). Elevated cyclic nucleotide levels are known to

downregulate the expression of cytokines (40-42), so we tested this hypothesis in BEAS-2B cells. However, under the experimental conditions used, TNF- α -induced IL-8 generation was unaffected by SNP/zaprinast or forskolin/rolipram pretreatment. Similar data were reported for human mesangial cells which, upon stimulation with IL-1 β , secreted less IL-6 but undiminished amounts of IL-8 (39, 41). The regulation of IL-8 production in different airway epithelial cell types may vary. For example, Levine and colleagues could not demonstrate an IL-8 reduction in dexamethasone-pretreated, TNF- α -stimulated BEAS-2B cells (40), whereas corticosteroids were active in TNF- α -activated A549 and primary human epithelial cells (17). It is clear that several stimuli and AEC types must be studied to resolve the relationship between cyclic nucleotides and epithelial cell cytokine secretion.

An appreciation of the PDE isoenzyme pattern in AEC is of obvious importance for the design of new therapeutic strategies. For example, all pulmonary and inflammatory cell types studied possess a PDE4, and therefore PDE4 inhibition will result in a broad cAMP accumulation and exert a generalized anti-inflammatory and bronchodilative effect. As for cGMP, it may be of interest that inhibition of PDE5 would affect AEC and airway smooth-muscle cells but not pulmonary endothelial cells because their cGMP degradation is governed by PDE2 (8, 9, 18). Differences in the PDE isoenzyme spectrum among pulmonary cell types therefore will allow a better targeting of cyclic nucleotide-based therapies.

The interpretation of our study is limited for several reasons: (1) The data show differences between the different types of AEC examined (porcine cells, human cells, BEAS-2B cell line). Primary cultures of human AEC are the cell type of major interest and therefore these cells were included in the present study. (2) Human airway epithelial cells were grown submerged in the medium; it is well known that this can affect cell differentiation. The relevance of our *in vitro* findings to airway epithelium *in vivo* therefore remains to be established. Growth of AEC at conditions of liquid-air interphase for prolonged time periods will probably result in a better model of airway epithelium. (3) An airway epithelial cell line and primary cultures of large airway epithelium were used. For an exact analysis of airway epithelial cell dysfunction in clinical disorders, it would be desirable to study the contributions made by epithelial cells of small airways of human origin. Isolation and culture of these cells, however, is difficult, and therefore the applicability of the data presented to human airway disease is not clear.

In summary, human AEC express PDE isoenzymes 1, 4 (variants 4A5, 4C1, 4D1, 4D2, and 4D3), 5, and 7, whereas BEAS-2B cells and porcine AEC show similar profiles but lack PDE1. Enhanced cAMP levels substantially increased and enhanced cGMP level reduced epithelial PGE₂ generation, whereas IL-8 and 15-HETE synthesis were not affected. Currently available therapies for inflammatory airway disease are nonspecific and not free of side effects. We suggest that cyclic nucleotide-based treatments that rely on differences in the PDE isoenzyme spectrum among pulmonary cell types could increase selectivity and reduce the side-effect profile of existing therapies. The

Identification of the PDE-Isoenzyme pattern in AEC may contribute to this aim.

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Phosphodiesterase expression in human epithelial cells

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Wright, Lyndon C., Joachim Seybold, Annette Robichaud, Ian M. Adcock, and Peter J. Barnes. Phosphodiesterase expression in human epithelial cells. *Am. J. Physiol.* 275 (Lung Cell. Mol. Physiol. 19): L694-L700, 1998.—Epithelial cells play a critical role in airway inflammation and have the capacity to produce many inflammatory mediators, including bioactive lipids and proinflammatory cytokines. Intracellular levels of cAMP and cGMP are important in the control of inflammatory cell function. These cyclic nucleotides are inactivated via a family of phosphodiesterase (PDE) enzymes, providing a possible site for drug intervention in chronic inflammatory conditions. We studied the expression of PDE activity in an epithelial cell line (A549) and in primary human airway epithelial cells (HAECs). We measured PDE function using specific inhibitors to identify the PDE families present and used RT-PCR to elucidate the expression of PDE isogenes. Both A549 cells and HAECs predominantly expressed PDE4 activity, with lesser PDE1, PDE3, and PDE5 activity. RT-PCR identified HSPDE4A5 and HSPDE4D3 together with HSPDE7. Inhibition of PDE4 and PDE3 reduced secretion by these cells. Epithelial PDE may be an important target for PDE4 inhibitors in the development of the control of asthmatic inflammation, particularly when delivered via the inhaled route.

phosphodiesterase type 3; phosphodiesterase type 4; inflammation; granulocyte-macrophage colony-stimulating factor; airway epithelial cells

CYCLIC NUCLEOTIDES play a key role in controlling epithelial cell functions such as electrolyte transport, ciliary motility, and cytokine production (12, 30). The rate of breakdown of cAMP and cGMP is controlled by a specialized superfamily of hydrolytic enzymes called cyclic nucleotide phosphodiesterases (PDEs). Several families of PDE enzyme have now been identified and are classified according to differing substrate specificity, regulatory characteristics, and sensitivities to selective inhibitors (4). There are at least seven recognized families of PDE, and most of these are known to contain at least two different genes. Additionally, for many of these genes, more than one splice variant is expressed (7). The availability of specific inhibitors has meant that the most widely studied families are PDE3, PDE4, and PDE5 (5, 20). There has been particular interest in PDE4 because it is predominant in inflammatory cells such as mast cells (2), monocytes (34), macrophages (31), eosinophils (11), and T cells (27). This suggests that PDE4 inhibitors may be useful in the treatment of several inflammatory and allergic diseases including atopic dermatitis (24), arthritis (25), multiple sclerosis (28), and bronchial asthma (13). Several PDE4 inhibitors have been shown to attenuate cytokine release from inflammatory cells and inhibit activated inflammatory cells (11, 13, 14).

It has become increasingly apparent that airway epithelial cells play a key role in the initiation and maintenance of the airway inflammatory response. Epithelial cells are not only passive barrier-target cells but also play an integral role in the pathophysiology of airway diseases through the release of multiple inflammatory mediators, including prostaglandins and 15-hydroxyicosatetraenoic acid (HETE) (23), and the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) (10), interleukin (IL)-1 (1), IL-8 (16), and regulated on activation normal T cells expressed and secreted (RANTES) (6). Because PDE4 inhibitors block the release of proinflammatory cytokines in inflammatory cells, they may also have an effect on epithelial cells.

One of the problems encountered in clinical studies with PDE4 inhibitors is the relatively high incidence of side effects such as nausea, vomiting, and headaches (3). Delivery of a PDE4 inhibitor topically by inhalation may avert the systemic side effects. However, little is known about the expression of PDE on airway epithelial cells. We have therefore investigated the expression of PDEs in cultured epithelial cells and in a human epithelial cell line, A549. Because at least four human PDE4 genes have now been identified (4, 20), we have also studied which genes are expressed in these cells.

MATERIALS AND METHODS

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Poole, UK).

Primary cells. Human epithelial cells were obtained from normal human lung donors and cultured as previously described (16). The sections of tracheal tissue were carefully cleaned and stored overnight on ice bathed in saline containing antibiotic penicillin (1 U/ml), streptomycin (0.1 mg/ml), and the antifungal agent amphotericin B (5 µg/ml). The bathing solution also contained pronase (1%) to increase epithelial cell shedding from the basement membrane. The following day, sections were lavaged for 60 min to dissociate the epithelial cells. To increase the yield of cells, the trachea could then be left in fresh bathing solution for a further 12 h before being washed. Cells were filtered through a sterile muslin gauze to remove mucus and debris and were seeded (0.5×10^5 cells) into six-well plates (Costar) for culture at 37°C and 5% CO₂ in a humidified incubator in supplemented Ham's F-12 medium (16).

A549 cells. The human epithelial-like (type II pneumocyte) cell line A549 (ATCC, Rockville, MD) was cultured in DMEM supplemented with 10% FCS, penicillin (1 U/ml), streptomycin (0.1 mg/ml), L-glutamine (2 mM), and amphotericin B (5 µg/ml) in a humidified incubator (37°C, 5% CO₂) as a stock culture in T-75 flasks (Costar). Confluent cultures were split and seeded into six-well plates at a density of 1×10^5 cells/well and cultured to confluence before use.

PDE assay. The complement of PDE isoenzymes was determined for each cell type by assaying PDE activity and

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utilizing specific inhibitors and reaction conditions known to distinguish specific isoenzymes. The activity assay measures the breakdown of [³H]cAMP or [³H]cGMP (Amersham, Little Chalfont, UK) to the corresponding labeled monophosphate by PDE and the subsequent dephosphorylation by alkaline phosphatase with a modification of the method of Thompson and Appleman (32). Reactions were performed at 37°C in an assay cocktail including 1 μM cAMP (including ~280,000 dpm [³H]cAMP; Amersham), [8-¹⁴C]adenosine (~6,000 dpm; Amersham), 0.25 U alkaline phosphatase, 20 mM triethanolamine (pH 8.0), 1 mM EGTA, 5 mM magnesium acetate, 5 mM dithiothreitol (DTT), 500 μg/ml of BSA and, where appropriate, any drug being used. To a 270-μl cocktail, 30 μl of enzyme were added to initiate the reaction. After a period of time predetermined by the number of cells required to utilize no more than 25% of the substrate, the reaction was stopped by the addition of 1 ml of a solution of Dowex AG 1-X8 anion exchanger-methanol-water (1:2:1). The preparation was then vortex mixed for 30 min at 4°C before centrifugation at 12,000 g, 4°C for 5 min. Aliquots of 700 μl were taken from each tube and counted (~60% efficiency) in 2 ml of aqueous counting scintillant (ACS II, Amersham). Assays for cGMP hydrolysis were performed by substituting for cold and labeled cAMP in the assay cocktail.

Assays were performed on soluble (crude cytosolic) and particulate (crude microsomal) cellular fractions prepared by hypotonic lysis of cells at 4°C in lysis buffer (10 mM MOPS, pH 7.4, 1 mM EGTA, 2 mM magnesium acetate, and 5 mM DTT), and protease inhibitors (100 μM leupeptin, 100 μg/ml of bacitracin, 2 mM benzamide, 100 μM phenylmethylsulfonyl fluoride, and 20 μg/ml of soybean trypsin inhibitor). After 50 min, the cell lysate was centrifuged at 4°C and 45,000 g for 30 min. The resulting supernatant was decanted and diluted in lysis buffer to the required cell equivalent per milliliter and used as a source of soluble enzyme. The pellet was resuspended at the required cell equivalent per milliliter and used as a source of membrane-associated enzyme.

Determination of PDE isoenzyme activities. Activities for individual PDE isoenzymes in A549 cells and human airway epithelial cells (HAECs) were determined by comparing total lysate hydrolytic activity in assay buffer alone with that seen in the presence of isoenzyme-specific inhibitors or allosteric modulators. Hydrolysis of cAMP and cGMP by soluble and particulate PDEs was assessed in the presence of 2 mM Ca²⁺ + 50 U calmodulin to unmask PDE1 activity. EGTA (1 mM) to eliminate residual PDE1 activity induced by Ca²⁺ in lysates (5 μM cGMP with or without 30 μM ORG-9935; Organon, Edinburgh, UK) was used to unmask PDE2 and PDE3 activity (19); 1 mM EGTA with or without 50 μM rolipram (Alexis, Nottingham, UK) was used to inhibit PDE4 activity; 1 mM EGTA (with or without 30 μM zaprinast) was used to inhibit PDE5 activity; and 500 μM 3-isobutyl-1-methylxanthine (IBMX) was used for inhibition of all PDE activity except PDE7 (17).

RNA isolation and RT-PCR. We designed forward and reverse PCR primers according to primary sequence data relating to the isoforms of PDE4 and PDE7 found in the GenBank database. The following primer sequences were constructed, with annealing temperature and product size in parentheses: HSPDE4A5 sense 5'-AAG AGG AGG AAG AAA TAT CAA TCG-3' and antisense 5'-TTA CAG CAA CCA CCA ATY CTT CCC-3' (67°C, 272 bp); HSPDE4B sense 5'-ACG CCA GCC TCA GCT ATT AA-3' and antisense 5'-CAJ CCG TCG CTT ACT GCT TTA-3' (62°C, 363 bp); HSPDE4C sense 5'-GGA CTT GCC TGA CAG TGA ACT-3' and antisense 5'-AGA AGA ACA CCA GGG CAT CGT-3' (71°C, 335 bp); and HSPDE4D sense 5'-GCA AGA TCG AGC ACC TGG CAA-3'

and antisense 5'-CGG TTA CCA GAC AAC TCT GCT-3' (68°C, 515 bp).

For HSPDE7 the primer sequences used were sense 5'-GGA CGT CCG AAT TAA GCAAGC-3' and antisense 5'-TCC TCA CTG CTC GAC TGT TCT-3' (59°C, 285 bp).

Extraction of RNA was performed after the modified guanidium acid-phenol method of Chomczynski and Sacchi (9). Genomic DNA contamination was reduced by 1:1 phenol-chloroform extraction and ethanol precipitation. Human lung total RNA extracted from 100 mg of donor tissue was used as a positive control. Sample RNA was quantified by spectropho-

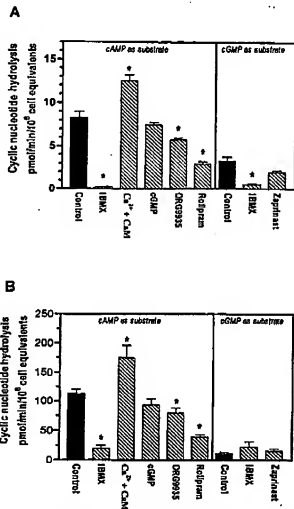


Fig. 1. Cyclic nucleotide hydrolysis by airway epithelial cell cytosolic phosphodiesterase (PDE). Hydrolysis in cytosolic lysate from human airway epithelial cells (HAECs; A) and A549 cells (B) in presence of selective inhibitors and allosteric modulators. Data are means \pm SE of 5 separate determinations. 3-Isobutyl-1-methylxanthine (IBMX) inhibits all PDEs except PDE7; control group shows mainly activity of PDE3 and PDE4 (cAMP substrate) or mainly PDE5 (cGMP substrate); Ca²⁺ (2 mM) + calmodulin (CaM; 50 U) stimulates PDE1 activity; cGMP (5 μM) stimulates PDE2 and inhibits PDE3; ORG-9935 (30 μM) inhibits PDE3; rolipram (50 μM) inhibits PDE4; and zaprinast (30 μM) inhibits PDE5. Significantly different from basal PDE activity. **P* < 0.05.



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ometry (absorbency 260 nm), and 1 μ g was reverse transcribed to cDNA in PCR buffer with 8 U avian myeloblastosis virus reverse transcriptase (Promega, Southampton, UK) and 0.2 μ g of random hexamers (Pharmacia, Uppsala, Sweden) in a final volume of 20 μ l. Then, the reaction mixture containing the cDNA was diluted to 100 μ l and stored at -70°C. PCR reactions were performed on 5 μ l of the cDNA with a Hybaid Oranigene thermal cycler (Hybaid, Middlesex, UK) in a final reaction volume of 25 μ l in the presence of 0.5 U of *Taq* DNA polymerase. Twenty-six to forty cycles were used, with a denaturing step at 90°C for 30 s, followed by the specific primer annealing temperature and an extension step at 72°C for 1 min. The PCR products were then separated

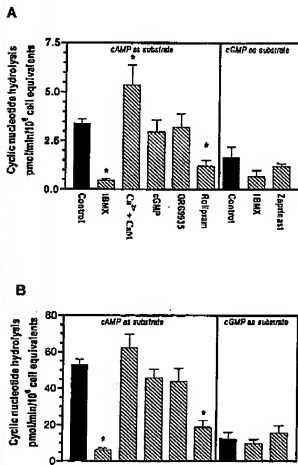


Fig. 2. Cyclic nucleotide hydrolysis by airway epithelial cell microsomal PDE. Hydrolysis in microsomal lysate from HAECs (A) and A549 cells (B) in presence of selective inhibitors and allosteric modulators. Data are means \pm SE of 5 separate determinations. IBMX inhibits all PDEs except PDE1; EGTA (1 mM) shows mainly activity of PDE3 and PDE4 (cAMP substrate) or mainly PDE5 (cGMP substrate); Ca²⁺ (2 mM) + cAMP (50 U) stimulates PDE1 activity; cGMP (5 μ M) stimulates PDE2 and inhibits PDE3; ORG-9333 (30 μ M) inhibits PDE3; roflumilast (50 μ M) inhibits PDE4; and zaprinast (30 μ M) inhibits PDE5. Significantly different from basal PDE activity. * $P < 0.05$.

□ Soluble
▨ Microsomal

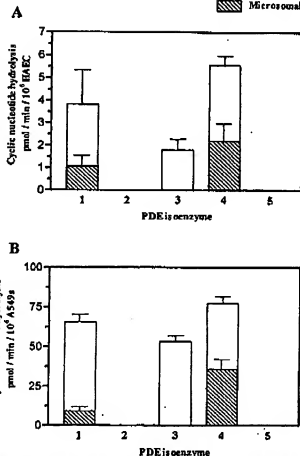


Fig. 3. PDE isoenzyme activities resolved in airway epithelial cells. According to assay criteria applied, HAECs (A) and A549 cells (B) displayed activities that may be attributed to PDEs 1, 3, and 4, whereas only HAECs displayed activity, suggesting presence of PDE5. No significant hydrolytic activity indicative of PDE2 in either cell type was found.

according to size on a 1.5% agarose gel containing ethidium bromide and visualized against a 1-kb ladder (GIBCO, Paisley, UK) over an ultraviolet light source.

To check that the desired target gene was being amplified, the product of each primer pair was cloned into pGEM5z (Promega), and double-stranded sequencing was performed with the Sequenase II kit (Amersham) to verify product identity.

GM-CSF Assay. Immunoreactive GM-CSF release from A549 cells and HAECs into culture medium (CM) was measured with an ELISA (Pharmingen, San Diego, CA), giving a lower detection limit of 15 pg/ml. The standard curves utilized for quantification were constructed with human recombinant GM-CSF (Genzyme, Cambridge, MA). Cells were grown in monolayers on six-well plates. When confluence was attained, the monolayers were washed to remove debris and nonadherent cells. Monolayers were then maintained for 24 h with fresh CM (1.0 ml) containing either drug or a volume equivalent of vehicle. The effect of PDE inhibitors

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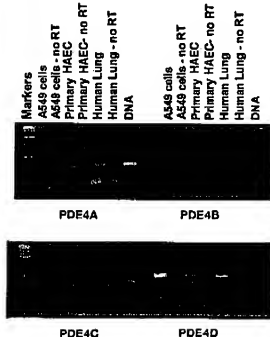


Fig. 4. Expression of multiple HSPDE4 isoform mRNAs in airway epithelial cells. A representative ethidium bromide-stained agarose gel ($n = 3$ experiments) of HSPDE4 PCR products amplified from HAEC and A549 cell cDNA was obtained by reverse transcribing 1 μ g of total cell RNA and subjecting this to PCR of 24–40 cycles in the presence of specific primers. Amplified product was separated by electrophoresis and visualized by ethidium bromide staining. Genomic contamination was controlled for by processing sample RNA in absence of reverse transcriptase (no RT), and total lung cDNA was used as a positive control.

(50 μ M rolipram, 30 μ M ORG-9395, and 500 μ M IBMX) on GM-CSF production was assessed with control cultures and cultures in which GM-CSF production was stimulated by 1 ng/ml of human recombinant IL-1 β (R&D Systems, Oxford, UK). PDE inhibitors were added to the CM 1 h before IL-1 β stimulation. After 24 h, CM was aspirated and assayed immediately for GM-CSF. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability test was then performed on the monolayers to control for potential PDE inhibitor-mediated cytotoxicity (8).

Analysis of data and statistics. All data are presented as means \pm SE of n independent experiments. Statistical analysis was performed on nontransformed data with analysis of variance, followed by a Bonferroni adjustment when multiple comparisons were made against a single mean. $P < 0.05$ was considered significant, and the null hypothesis was rejected.

RESULTS

Identification of functional PDEs. Figures 1 and 2 show the amount of cAMP and cGMP hydrolyzed by PDEs in primary HAECs and the type II pneumocyte adenocarcinoma epithelial-like A549 cells. Hydrolytic activity was measured under conditions selected to unmask the proportion of hydrolysis attributable to the different members of the PDE superfamily present in the cytosolic (Fig. 1) and crude microsomal (Fig. 2) fractions.

The nonselective PDE inhibitor IBMX significantly suppressed cAMP and cGMP hydrolytic activity in the microsomal fractions and almost all of that associated with the cytosolic fractions, suggesting that the PDEs are the unique means of cyclic nucleotide catabolism in both cell types. However, a percentage of activity, albeit small, that was not blocked by IBMX remained in both HAECs and A549 cells. We reasoned that this could suggest the presence of small amounts of PDE7 in these cells, a speculation further supported by the RT-PCR data obtained (see Fig. 5). In the presence of the allosteric modulator calmodulin and an excess of Ca^{2+} , cAMP hydrolysis increased above that seen in the presence of the chelating agent EGTA alone by $39.1 \pm 10.3\%$ and by $7.0 \pm 5.6\%$ for cytosolic and microsomal HAEC PDEs. Similarly, in A549 cells, the increase was $36.4 \pm 9.4\%$ for cytosolic PDE and $6.2 \pm 1.4\%$ for microsomal PDE. This indicates the presence of cytosolic PDE1 in both primary cells and A549 cells. The addition of cGMP in the presence of EGTA caused no significant difference in cAMP hydrolysis in HAECs, which, taken alone, suggests that PDE2 is absent. In the presence of the selective PDE3 inhibitor ORG-9395, cytosolic HAEC cAMP hydrolysis was inhibited significantly ($23.8 \pm 4.9\%$), whereas microsomal hydrolysis was not significantly inhibited. Taken together, these

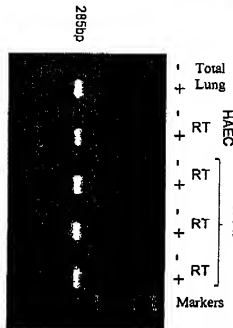


Fig. 5. Expression of HSPDE7 mRNA in airway epithelial cells. A representative ethidium bromide-stained agarose gel ($n = 3$ experiments) of HSPDE7 PCR products amplified from HAEC and A549 cell cDNA was obtained by reverse transcribing 1 μ g of total cell RNA and subjecting this to PCR of 24–40 cycles in the presence of specific primers. Amplified product was separated by electrophoresis and visualized by ethidium bromide staining. Genomic contamination was controlled for by processing sample RNA in presence (+RT) or absence (-RT) of reverse transcriptase or total lung cDNA was used as a positive control.



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data suggest the presence of cytosolic but not membrane-associated PDE3. Likewise, in the A549 cell line, cGMP failed to induce activity in either subcellular fraction, suggesting that PDE2 is absent. ORG-9935 caused a significant ($30.0 \pm 2.6\%$) inhibition in the cytosol, suggesting the presence of PDE3 activity. Rolipram caused a substantial reduction in cAMP hydrolyzing activity in HAECs, with a 75.0 ± 6.2 and $62.1 \pm 4.1\%$ fall in cytosolic and microsomal activity, respectively. Likewise, in the A549 cells, cAMP hydrolysis was reduced in the cytosolic and microsomal fractions by 67.6 ± 7.0 and $73.1 \pm 4.3\%$, respectively. This suggests that PDE4 was present in all subcellular fractions tested. In HAECs, cGMP was hydrolyzed by both cytosolic and microsomal cell fractions. This activity was inhibited by 30.2 ± 5.5 (cytosolic) and $38 \pm 15.7\%$ (microsomal) in the presence of $50 \mu\text{M}$ zaprinast, although this was not significant. This suggests that PDE5 was not the only source of cGMP metabolism.

By pooling these results, we are able to produce the PDE isoenzyme activity profile for HAECs and A549 cells shown in Fig. 3.

RT-PCR. Figure 4 is a representative photograph from three separate experiments of an ethidium bromide-stained gel showing the results of the RT-PCR amplification, the specific sequences corresponding to HSPDE4A, HSPDE4B, HSPDE4C, and HSPDE4D3 isoforms of PDE4. The primary HAECs and the A549 cells showed the same PDE4 profile, both cell types expressing PCR products corresponding to the message for the HSPDE4A and HSPDE4D isoforms but not for HSPDE4B or HSPDE4C. To complete the profile of known PDEs for these cell types, PCR was performed to check for HSPDE7 mRNA (Fig. 5; representative of 3 experiments), and both cell types expressed a strong signal.

GM-CSF ELISA. Figure 6 shows the release of GM-CSF from HAECs and A549 cells after culture for 24 h. PDE inhibitors failed to palliate this basal GM-CSF output. The presence of IL-1 β at 1 ng/ml causes a significant increase in the release of immunoreactive GM-CSF by both cell types. Treatment of stimulated cells with ORG-9935 or rolipram caused a significant decrease in GM-CSF output. ORG-9935 reduced GM-CSF release to baseline levels. IBMX, which gives inhibition of all PDE activity in these cells, obliterated IL-1 β -mediated GM-CSF release. The MTT assay revealed that PDE inhibitor-mediated cytotoxicity cannot account for the reduction in GM-CSF secretion.

DISCUSSION

This study characterized the PDE isoforms found in primary HAECs and the epithelial cell line A549. Previous studies characterized PDEs in bovine tracheal epithelial cells (22) and in human airways (21), but this is the first characterization of PDE in primary HAECs. Our results show the major cytosolic PDE activity in primary HAECs to be PDE4, with lesser activities of PDE3 and PDE1. A similar profile, but of lower activity, was seen for the enzyme associated with the membrane

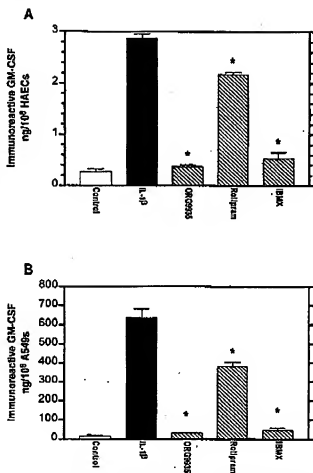


Fig. 6. Selective PDE inhibitors partially abrogate granulocyte-macrophage colony-stimulating factor (GM-CSF) release from airway epithelial cells (HAECs (A) and A549 cells (B)). Data are means \pm SE of 5 separate experiments. Control cells were untreated, and PDE inhibitors had no effect on basal GM-CSF secretion. All other cultures were treated with interleukin (IL)-1 β (1 ng/ml) after 1-h pretreatment with either 30 μM ORG-9935, 50 μM rolipram, or 500 μM IBMX. GM-CSF release is significantly less than that seen with IL-1 β alone. * $P < 0.05$.

subcellular fraction. The A549 cell line presented activities for PDE4, PDE3, and PDE1, with a similar quantitative subcellular distribution but with activity ~ 10 -fold higher than that seen in primary HAECs. This increased activity may be a consequence of the transformed nature of the A549 cells and suggests that these cells may not be suitable as a model for studying PDE induction patterns in airway epithelial cells. We have shown that PDE4 is the main enzyme responsible for cAMP hydrolysis in HAECs, and this is in broad agreement with findings in bovine tracheal epithelial cells (23) and human airway (21), although Rabe et al. (21) showed that the activity is associated with the cellular milieu in peripheral airways. This mixture of cell types may account for the discovery of significant amounts of PDE5 activity in whole peripheral airways.

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in contrast to our results in which PDE5 activity, as defined by zaprinast-mediated inhibition, did not attain significance. PDE4 has also been reported to be present as a major hydrolytic activity in both the cytosolic and membrane fractions of several cell types, including eosinophils (14), T lymphocytes (12), monocytes (26), and cells in the brain (18), myocardium (15), and trachea (33). PDE2 was absent from airway epithelial cells, and this suggests no role for this isoenzyme in regulating cellular function in the basal state. The small degree of hydrolytic activity found in all fractions that was not inhibited by IBMX may be due to a low background level of the methylxanthine-resistant PDE7 (17). Although this was supported by the identification of HSPDE7 mRNA in these cells, the negligible amount of activity suggests that this PDE does not perform a significant hydrolytic function in the basal state in these cell types. Message corresponding to PDE7 is also present in other inflammatory cells, including T cells (12), and is present in abundance in other tissues, but elucidation of the functional role, if any, played by this increasingly ubiquitous isoenzyme must await the development of specific inhibitors.

Using RT-PCR, we demonstrated that HSPDE4A5 and HSPDE4D3 were expressed in both primary HAECs and A549 cells, whereas HSPDE4B and HSPDE4C were absent in the basal state. To briefly summarize the data currently available concerning PDE4 mRNA subtype distribution in mammalian cells and tissues, PDE4A, PDE4B, and PDE4D appear to be fairly ubiquitous, with only PDE4C showing a more discrete peripheral distribution (20). The pattern of expression we have found in HAECs is similar to that observed in the T lymphocyte cell line Jurkat 30 and lung carcinomas (20). The significance of differential PDE subtype expression remains unknown, and a more discrete cell and tissue distribution of PDE may become apparent with elucidation of the expression of the spliced variants of PDE subtypes.

We have shown that IL-1 β promotes GM-CSF release from airway epithelial cells and that inhibition of PDE significantly reduces the liberation of this cytokine from these cells into their surrounding media. Interestingly, whereas hydrolysis experiments show that PDE3 is present in less significant amounts than PDE4 in both cell types, the PDE3-selective inhibitor ORG-9935 abrogates all GM-CSF release to baseline levels in these cells. Indeed, nonspecific inhibition of all major PDE activity by IBMX reduced release of this cytokine by no more than that achieved by inhibition of PDE3 alone. This suggests a key role for discrete PDE isoenzymes in control of cytokine release from airway epithelial cells, and the palliating effect of PDE inhibitors on GM-CSF release from these cells indicates that these drugs may be useful in asthma.

The demonstration that PDE4 predominates in airway epithelial cells suggests that these cells may be an important target for PDE4 inhibitors in the treatment of asthma and other inflammatory diseases of the airways. There is increasing evidence that airway epithelial cells in asthma produce and express cyto-

kines such as GM-CSF (10), IL-1 β (1), RANTES (6), and monocyte chemoattractant-1 (29). PDE4 inhibitors may inhibit the release of cytokines from these cells through an increase in cAMP concentrations. This may be important in the clinical development of these drugs for asthma therapy because a major problem appears to be systemic side effects such as nausea and headache. Delivering these drugs via inhalation may make it possible to suppress inflammation and minimize these side effects. In the future, more selective PDE4 inhibitors may be developed (20), and possibly these will have fewer side effects than existing nonselective drugs. The identification of HSPDE4A5 and HSPDE4D3 in airway epithelial cells suggests that more selective inhibition might be possible.

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Regulatory roles of adenylate cyclase and cyclic nucleotide phosphodiesterases 1 and 4 in interleukin-13 production by activated human T cells

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Abstract

We studied the activities of 3',5'-adenosine-cyclic monophosphate (cAMP)-synthesizing adenylate cyclase (AC) and cAMP-hydrolyzing cyclic nucleotide phosphodiesterase (PDE) in phytohemagglutinin (PHA)- or anti-CD3 plus anti-CD28-stimulated human T cells, and examined their roles in interleukin-13 (IL-13) production. The AC inhibitor MDL 12,330A [*cis*-N-(2-phenylcyclopentyl)azacyclotride-1,4n-2-amine hydrochloride] completely blocked PHA- or anti-CD3/CD28-induced IL-13 production. The PDE 1 inhibitor 8-methoxymethyl-3-isobutyl-1-methylxanthine or the PDE4 inhibitor rolipram partially inhibited IL-13 production, and the addition of both resulted in 100 or 85% inhibition in PHA- or anti-CD3/CD28-stimulated T cells, respectively. AC in T cells was transiently activated 5 min after stimuli, followed by the transient activation of PDE4 at 30 min. PDE1 activity, undetectable in resting T cells, was detected 3 hr after stimuli, and then increased gradually. Although PDE1-, 2-, 3-, and 4-independent PDE activity was low ($\leq 15\%$ of total), it began to increase 3 hr after anti-CD3/CD28; the increase was blocked by PDE7 antisense oligonucleotide, and such an increase was not induced by PHA. PHA or anti-CD3/CD28 induced PDE1B mRNA expression, undetectable in resting T cells. PDE4 mRNA level in T cells was not altered by either stimulus. PDE7 mRNA expression was detected in resting T cells, and was enhanced by anti-CD3/CD28, but not by PHA. The cAMP level of T cells increased 5 min after stimuli, returned to the basal level at 2 hr, and then continued to decrease. These results suggest that PHA or anti-CD3/CD28 initially (≤ 5 min) increases cAMP in T cells via AC, then reverses the increase via PDE4 (≤ 2 hr), and in the later phase (> 2 hr) further decreases cAMP via PDE1. Both the time-dependent increase and decrease of cAMP may be required for IL-13 production. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Phytohemagglutinin; Anti-CD3; Anti-CD28; Interleukin-13; Adenylate cyclase; Cyclic nucleotide phosphodiesterase

1. Introduction

IL-13 is a B cell-stimulatory cytokine that induces IgE and IgG4 production in B cells by isotype switching [1], and may be involved in the pathogenesis of certain allergic

diseases such as atopic dermatitis or asthma [2,3]. This cytokine is produced mainly by T cells [1], and the production is induced by PHA or anti-CD3 plus anti-CD28 antibodies [4,5]. However, signaling pathways involved in IL-13 production have not been identified definitively. It was reported recently that cAMP-elevating agents inhibit allergen-induced IL-13 production in human T cells and basophils [6,7]. It also has been reported that prolonged and excessive cAMP signal down-regulates the other T cell functions such as proliferation, IL-2 or IL-4 production, or IL-2 receptor expression [6,8–10]. Cellular cAMP levels are controlled by AC, which synthesizes cAMP, and by PDE, which hydrolyzes cAMP [11]. Thus, cAMP accumulation is manifested by AC-stimulating agents, such as prostaglandin E₂ or forskolin [8], and by PDE inhibitors, such as theophylline [6].

Previous studies especially support the requirement of

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Abbreviations: AC, adenylate cyclase; AP, activator protein; AS-O, antisense oligonucleotide; cAMP, 3',5'-adenosine-cyclic monophosphate; cGMP, 3',5'-guanosine-cyclic monophosphate; EHN, *erythro*-9-(2-hydroxy-3-oxo)-adenine; GAM, goat anti-mouse IgG polyclonal antibody; IBMX, 3-isobutyl-1-methylxanthine; IL-13, interleukin-13; 8-methoxymethyl-IBMX, 8-methoxymethyl-3-isobutyl-1-methylxanthine; NS-O, non-sense oligonucleotide; PCR, polymerase chain reaction; PDE, cyclic nucleotide phosphodiesterase; PHA, phytohemagglutinin; RT, reverse transcription; and TCR, T cell receptor.

PDE for IL-13 production [6,7]. PDE may possibly reduce the cellular cAMP level and thus prevent the inhibitory effect of cAMP accumulation on IL-13 production. PDE is an isozymic family composed of various subtypes classified on the basis of substrate specificity, inhibitor specificity, and sequence homology [7]. The previous paper suggests that cAMP-specific PDE (PDE4) may be involved in IL-13 production by human T cells [7].

In this study, we investigated the involvement of AC and PDE in PHA- or anti-CD3 plus anti-CD28-induced IL-13 production of T cells. We found that AC and PDE activities were time-dependently up- and down-regulated after the stimuli, and that both were required for IL-13 production.

2. Materials and methods

2.1. Reagents

The purified form of *Phaseolus vulgaris* PHA, actinomycin D, cycloheximide, EHNA, and affinity-purified GAM were purchased from the Sigma Chemical Co. Rolipram, 8-methoxymethyl-IBMX, cilostamide, MDL 12,330A [*cis*-N-(2-phenylcyclopropyl)azacyclotridec-1-en-2-amine hydrochloride], SQ22536 [9-(tetrahydro-2'-furyl)adenine], 2',5'-dideoxyadenosine, vinpocetine, Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone], milrinone, IBMX, 8-bromo-cAMP, and H-89 [N-[2-(*p*-bromocinnamyl)amino]ethyl]-5-isouquinolinesulfonamide] were obtained from Calbiochem, and were dissolved in dimethyl sulfoxide as 10 mM stock solutions and kept in the dark until used. Anti-CD3 monoclonal antibody (OKT3, murine IgG2a) and anti-CD28 monoclonal antibody (clone 9.3, murine IgG2a) were purchased from Becton Dickinson.

2.2. Preparation of human T cells

Blood was taken from five healthy Japanese volunteers (two men and three women, age 43.6 ± 13.6 years (mean \pm SD)), who were informed of the objectives and methods of this study, and consented to participate. Peripheral blood mononuclear cells were isolated by centrifugation over Ficoll-Paque (Pharmacia) as described [12], and were allowed to adhere to plastic dishes for 1 hr at 37°. From the non-adherent cells, CD56⁺ cells were isolated by negative selection using immunomagnetic beads (Dyna) as described [13], and were incubated with neuraminidase-treated sheep erythrocytes as described [14]. From the rosette-forming cells, CD14⁺ and CD19⁺ cells were isolated by immunomagnetic negative selection, and were used as T cells. This T cell population was >98% CD3⁺, and the contamination of CD14⁺, CD19⁺, or CD56⁺ cells was <2%.

2.3. Measurement of IL-13 and cAMP

T cells ($2 \times 10^5/200 \mu\text{L}/\text{well}$) were cultured with $10 \mu\text{g}/\text{mL}$ of PHA or anti-CD3 plus anti-CD28 antibodies

(each $0.1 \mu\text{g}/\text{mL}$) in plates coated with GAM ($10 \mu\text{g}/\text{mL}$) as described [15], at 37° in an atmosphere of 5% CO₂ for 48 hr. T cells were cultured with medium alone in parallel. Each culture was performed in triplicate. The culture medium used was a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's Nutrient Mixture F-12 (Sigma), supplemented with 2.5 mM L-glutamine (Gibco/BRL). The activity of IL-13 in the culture supernatants was measured by an ELISA kit (Biosource). The sensitivity of the assay was 12 pg/mL. To analyze the inhibitory effects of various AC or PDE inhibitors on PHA- or anti-CD3/CD28-induced IL-13 production, these agents were added at various time points before, simultaneously, or after the stimuli. The percent inhibition of either stimulus-induced IL-13 production was calculated by the following equation: (IL-13 amount with stimulus - IL-13 amount with stimulus plus inhibitor) \div (IL-13 amount with stimulus - IL-13 amount with medium alone) $\times 100$ (%). To measure the intracellular cAMP level at various time points, medium was discarded and cells were lysed with ethanol, the lysates were centrifuged at 10,000 g for 10 min at 4°, and the supernatants were dried under vacuum. The dried samples were dissolved in acetate buffer (pH 5.8), and cellular cAMP contents were measured by an ELISA kit from Amersham. The sensitivity of the assay was 12 fmol/assay well. The cAMP level was presented as picomoles per 10^6 cells.

2.4. Measurement of PDE activity

T cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 $\mu\text{g}/\text{mL}$ of aprotinin, 1 $\mu\text{g}/\text{mL}$ of pepstatin, 1 $\mu\text{g}/\text{mL}$ of leupeptin, 15 mM benzamide, and 3.75 mM *B*-mercaptoethanol. PDE activity of the cell lysates was assayed as described previously [8,16] using 1 μM [2,8-³H]cAMP (30 Ci/mmol) (Amersham) as a substrate. The assays were performed in 40 mM Tris-HCl (final pH 8.0), 10 mM MgCl₂ in the presence of 0.2 mM CaCl₂ and 15 mM human brain calmodulin (Calbiochem) at 37° for 10 min, and PDE activity was presented as picomoles cAMP hydrolyzed per minute per milligram protein. Total PDE activity represented the activity in the absence of inhibitors; cGMP-inhibited PDE (PDE3) or cAMP-specific PDE (PDE4) activity was defined as the inhibition of the activity by 10 μM cilostamide (a specific PDE3 inhibitor) or by 10 μM rolipram (a specific PDE4 inhibitor), respectively, as described [17]. PDE activity in the presence of 1 mM EGTA (a calcium chelator) and without Ca²⁺/calmodulin was measured, and the difference from the total PDE activity was defined as Ca²⁺/calmodulin-dependent PDE (PDE1) activity as described [18,19]. PDE activity in the presence of 10 μM cGMP was measured, and the inhibition by 10 μM EHNA was defined as cGMP-stimulated PDE (PDE2) activity as described [19]. The PDE1, 2, 3-, and 4-independent PDE activity was defined as PDE activity measured in the presence of 8-methoxymethyl-IBMX, EHNA, cilostamide, and rolipram (each 10 μM).

Table 1
Primers used for RT-PCR

Genes	Primer sequences	Product size (bp)	Ref.
PDE1B	5'-GCC TCA TCA GCC GCT TCA AGA TTC C-3' 5'-GAA CTC CTC CAT TAG GGC CTT GG-3'	601	[19]
PDE4A	5'-AAC AGC CTG AAC AAC TNT AAC-3' 5'-CAA TAA AAC CCA CCT GAG ACT-3'	907	[17]
PDE4B	5'-AGC TCA TGA CCC AGA TAA GTC-3' 5'-ATA ACC ATC TTC CTG AGT GTC-3'	625	[17]
PDE4C	5'-TCG ACA ACC AGA GGA CTT AGG-3' 5'-GGA TAG AAG CCC AGG AGA AAG-3'	289	[17]
PDE4D	5'-CCC TTG ACT GTT ATC ATG CAC ACC-3' 5'-CCC TTG ACT GTT ATG CAC ACC-3'	262	[18]
PDE7	5'-GGA COT EGG AAT TAA GCA AGC-3' 5'-TCC TCA CTG CTC GAC TGT TCT-3'	285	[23]
β -Actin	5'-GGG TCA GAA GGA TTC CTA TG-3' 5'-GGT CTC AAA CAT GAT CTG GG-3'	268	[22]

2.5. Measurement of AC activity

The T cell lysate above was centrifuged at 23,600 g for 10 min at 4°. The pellet was used as a particulate fraction for AC assays as described [20,21]. The AC activity was measured at 37° for 10 min in 20 mM Tris-HCl (pH 7.4), 1 mM [α -³²P]adenosine triphosphate (30 Ci/mmol) (Amersham), 1 mM [³H]cAMP, 1 mM IBMX, 5 mM MgCl₂, 0.2 mM EGTA, 20 mM creatine phosphate, and 100 U/mL of creatine phosphokinase. AC activity was presented as picomoles cAMP formed per minute per milligram protein.

2.6. RT-PCR

T cells were incubated under the conditions indicated, and total cellular RNA was extracted using a mRNA purification kit (Pharmacia). cDNA was made from RNA samples using Moloney mouse leukemia virus reverse transcriptase (Gibco-BRL) as described previously [22]. PCR was performed using primer sets (Table 1) in the thermal cycler programmed at 93° for 1 min, 60° for 1 min, and 72° for 2 min for 35 cycles. The PCR products were analyzed by electrophoresis on 2.5% agarose gels and stained with ethidium bromide. The intensity of the band for the RT-PCR products was determined by densitometry (Hoefer Scientific Instruments). Negative controls included samples in which cDNA synthesis was performed in the absence of reverse transcriptase and samples in which no cDNA was added during PCR.

2.7. Statistical analyses

One-way analysis of variance with Scheffé's multiple comparison test was used for the data in Fig. 5. One-way analysis of variance with Dunnett's multiple comparison test was used for the data in Table 2. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Inhibitory effects of AC and PDE inhibitors on IL-13 production

To clarify the involvement of AC and PDE isotypes in the PHA- or anti-CD3/CD28-induced IL-13 production, T cells were preincubated with AC or PDE isotype-specific inhibitors before stimulation, and their inhibitory effects on IL-13 production were examined. In PHA-stimulated T cells, the AC inhibitor MDL 12,330A blocked IL-13 production completely ($IC_{50} = 0.1 \mu\text{M}$) (Fig. 1A). The other

Table 2
Inhibitory effects of cAMP-modulating agents on PHA- or anti-CD3/CD28-induced IL-13 production

Agents	IL-13 production (pg/mL)	
	PHA	Anti-CD3/CD28
None	1202 \pm 121	1452 \pm 186
IBMX (0.5 mM)	43 \pm 9* (98)	33 \pm 6* (99)
Vinpocetine (50 μM)	483 \pm 52* (61)	804 \pm 73* (45)
EHNA (10 μM)	1172 \pm 150 (3)	1430 \pm 151 (2)
Milrinone (10 μM)	1162 \pm 191 (3)	1420 \pm 153 (2)
RO-20-1724 (10 μM)	732 \pm 82* (44)	917 \pm 98* (37)
SQ22536 (10 μM)	58 \pm 9* (95)	81 \pm 9* (95)
2',5'-DDOEA (10 μM)	68 \pm 12* (95)	52 \pm 7* (97)
Forskolin (100 μM)	21 \pm 4* (99)	36 \pm 5* (98)
8-bcAMP (1 mM)	34 \pm 5* (98)	66 \pm 9* (96)

T cells from five different donors (two men and three women) were preincubated with AC or PDE inhibitors at indicated concentrations for 30 min, and were stimulated with PHA (10 $\mu\text{g/mL}$) or anti-CD3/CD28 (each 0.1 $\mu\text{g/mL}$) as described in the legend of Fig. 1. After 48 h, the culture supernatants were assayed for IL-13 by ELISA. Data are means \pm SEM ($N = 5$). The values in parentheses are the percent inhibition calculated by the equation described in "Materials and methods." The IL-13 amount with medium alone was $13 \pm 5 \text{ pg/mL}$ (mean \pm SEM, $N = 5$). Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; 2',5'-dideoxyadenosine; and 8-bcAMP, 8-bromo-cAMP. * Significantly different from controls with either stimulus alone ($P < 0.05$).

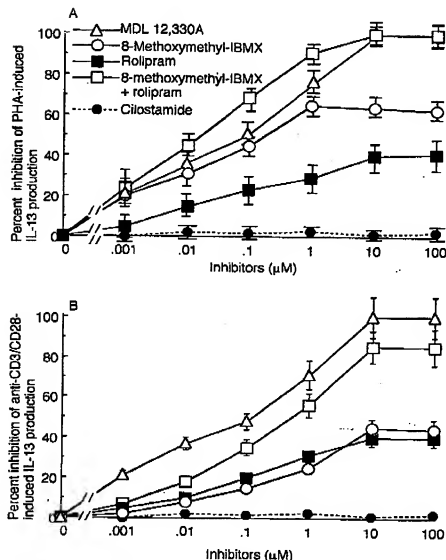


Fig. 1. Concentration-dependency for the inhibitory effects of AC or PDE inhibitors on IL-13 production. T cells from five different donors (two men and three women) were preincubated for 30 min with medium alone or with medium containing various AC or PDE inhibitors at the indicated concentrations in GAM-coated wells; then PHA (final concentration of 10 μg/mL) (A) or anti-CD3/CD28 (each 0.1 μg/mL) (B) was added. T cells were cultured with medium alone in parallel. After 48 hr, the amounts of IL-13 in the culture supernatants were measured by ELISA. Data are presented as percent inhibition calculated by the equation described in "Materials and methods." Values are means \pm SEM (N = 5). The amount of IL-13 without inhibitors was as follows: 13 \pm 5 pg/mL in medium alone, 1202 \pm 121 pg/mL in PHA alone, and 1452 \pm 186 pg/mL in anti-CD3/CD28 alone (mean \pm SEM, N = 5).

AC inhibitors, SQ22536 and 2',5'-dideoxyadenosine, also blocked the PHA-induced IL-13 production completely (Table 2). These results suggest the requirement of AC for PHA-induced IL-13 production. Among the PDE isotype-specific inhibitors, the PDE1 inhibitor 8-methoxymethyl-IBMX was the most inhibitory on IL-13 production (61% inhibition at 1 μM; Fig. 1A). The PDE4 inhibitor rolipram showed a smaller, but significantly inhibitory effect (38% inhibition at 10 μM). The usage of both 8-methoxymethyl-

IBMX and rolipram gave additive inhibitory effects, and completely blocked PHA-induced IL-13 production when added at 10 μM each. Another PDE1 inhibitor, vinpocetine, or the PDE4 inhibitor Ro-20-1724 also inhibited PHA-induced IL-13 production by a magnitude comparable to that of 8-methoxymethyl-IBMX or that of rolipram, respectively (Table 2). Neither the PDE3 inhibitor cilostamide (Fig. 1A) nor milrinone (Table 2) inhibited PHA-induced IL-13 production significantly. Likewise, the PDE2 inhibi-

tor EHNA did not have a significant inhibitory effect (Table 2). PHA-induced IL-13 production was blocked completely by the non-specific PDE inhibitor IBMX, the AC activator forskolin, or the cAMP analogue 8-bromo-cAMP (Table 2). These results indicate that a prolonged and excessive cAMP signal may suppress PHA-induced IL-13 production.

On the anti-CD3/CD28-induced IL-13 production (Fig. 1B, Table 2), the inhibitory effects of AC and PDE inhibitors were mostly comparable to those on PHA-induced IL-13 production; anti-CD3/CD28-induced IL-13 production was inhibited completely by AC inhibitors (IC_{50} of MDL 12,330A = 0.12 μ M), and was partially blocked by PDE1 or PDE4 inhibitors, but not by PDE2 or PDE3 inhibitors. The inhibitory effect of 8-methoxymethyl-IBMX on anti-CD3/CD28-induced IL-13 production (45% inhibition at 10 μ M) was slightly smaller than that on PHA-induced IL-13 production. The addition of both 8-methoxymethyl-IBMX and rolipram (each at 10 μ M) inhibited the anti-CD3/CD28-induced IL-13 production by 85%; however, the combination did not inhibit IL-13 production completely even with further increased concentrations, indicating the possible contribution of another PDE isotype(s). These results suggest that both PDE1 and PDE4 may be required for PHA- or anti-CD3/CD28-induced IL-13 production, and the contribution of each PDE isotype to the IL-13 production seems to be independent. Anti-CD3/CD28-induced IL-13 production may possibly involve the other PDE isotype(s). We then tried to clarify at which time AC, PDE1, or PDE4 is required after the stimuli.

3.2. Time-dependence for the effects of AC, PDE, and PDE4 inhibitors on IL-13 production

MDL 12,330A, rolipram, or 8-methoxymethyl-IBMX was added to T cells at various time points before, simultaneously with, or after the stimuli, and the inhibitory effects on IL-13 production were compared. In PHA-stimulated T cells, the inhibitory effect of each agent was most potent when added before the stimulus, and decreased with delayed addition after the stimulus (Fig. 2A). The addition of MDL 12,330A 10 min after PHA did not inhibit IL-13 production at all, and further delayed addition was not inhibitory either (data not shown). This suggests that AC may be required only in the very early phase (<10 min) of the IL-13 induction. When rolipram was added 2 hr after PHA, its inhibitory effect was mostly lost, although a weak inhibitory effect (about 8% inhibition) was detected by the addition at 8 hr. This indicates that PDE4 may be involved mainly in the first 2 hr of IL-13 induction, although it may also be required in the later phase. The inhibitory effect of 8-methoxymethyl-IBMX was not reduced by addition 2 hr after PHA; however, the inhibitory effect gradually decreased with further delayed addition. This indicates that PDE1 may be required for the later phase (>2 hr) of IL-13 induction. In anti-CD3/CD28-stimulated T cells (Fig. 2B), the time-dependence for the inhibition by AC, PDE1, and

PDE4 inhibitors was mostly equivalent to that in PHA stimulation. We then studied the kinetics of AC and PDE isozyme activities after the stimuli.

3.3. Kinetics for activities of AC and PDE isozymes

The AC activity of T cells rapidly increased, peaked at 5 min after PHA or anti-CD3/CD28 stimulation, and returned to the basal level at 1 hr (Fig. 3). Following the transient activation of AC, PDE4 activity increased and peaked at 30 min after either stimulus, and returned to the basal level at 2 hr (Fig. 4A). The transient activation of PDE4 was not blocked by actinomycin D or cycloheximide (Fig. 5A), indicating that the activation may not involve *de novo* protein synthesis. The activation of PDE4 was blocked by the cAMP-dependent protein kinase (protein kinase A) inhibitor H-89, indicating the involvement of protein kinase A. PDE1 activity was not detected in resting T cells, and was induced by PHA or anti-CD3/CD28 (Fig. 4B). PDE1 activity was detected at 3 hr after either stimulus, and gradually increased up to 24 hr. Although the PHA-induced PDE1 activity appeared to be slightly higher than that induced by anti-CD3/CD28, the difference was not significant. The PHA- or anti-CD3/CD28-induced PDE1 activity was suppressed by actinomycin D and cycloheximide (Fig. 5B), indicating the requirement of *de novo* protein synthesis. The induction of PDE1 was not blocked by H-89, indicating that protein kinase A may not be involved in the induction. PDE3 activity was not altered by either PHA or anti-CD3/CD28 (Fig. 4C). PDE2 activity was not detected in any of the control, PHA-, or anti-CD3/CD28-stimulated T cells over a 0- to 48-hr period (data not shown). At 24 hr, the sum of PDE1, 3, and 4 activities formed more than 85% of total PDE activity (Fig. 4D) in control, PHA-, or anti-CD3/CD28-stimulated T cells. However, a small proportion of PDE activity was insensitive to the inhibition by 8-methoxymethyl-IBMX, EHNA, cilostamide, and rolipram, i.e. independent of PDE1, 2, 3, and 4. In anti-CD3/CD28-stimulated T cells, the PDE1-, 2-, 3-, and 4-independent PDE activity (Fig. 4E) began to increase at 3 hr and peaked at 12 hr. Such an increase was not induced by PHA. At 12 hr, the proportion of PDE1-, 2-, 3-, and 4-independent PDE activity was 5, 2, or 15% of the total PDE activity in control, PHA- or anti-CD3/CD28-stimulated T cells, respectively. A recent paper proved the mRNA expression of cAMP-specific, rolipram-insensitive PDE (PDE7) in human peripheral T cells and indicated its enzymatic activity in these cells [23]. Since PDE7-specific inhibitor is not currently available, a 24-bp PDE7 antisense oligonucleotide (AS-O) (5'-CGGCAGCTGTGTAACACACTTCCAT-3') was synthesized [24] and was tested. As a control, a nonsense oligonucleotide (NS-O) containing a reversed sequence also was synthesized. In anti-CD3/CD28-stimulated T cells, the increase of PDE1-, 2-, 3-, and 4-independent PDE activity was blocked by PDE7 AS-O but not by PDE7 NS-O (Fig. 5C), suggesting that the activity may consist mostly of

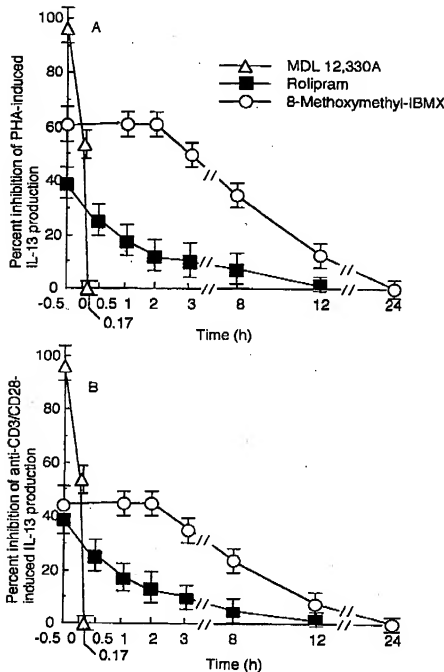


Fig. 2. Time-dependence for the inhibitory effects of AC or PDE inhibitors on IL-13 production. T cells from five different donors (two men and three women) were stimulated with PHA 10 μ g/mL (A) or anti-CD3/CD28 (each 0.1 μ g/mL) (B) in GAM-coated wells for 48 hr, and the amount of IL-13 in the culture supernatants was measured by ELISA. The time when the stimuli were added is defined as 0 hr. Rolipram or 8-methoxymethyl-IBMX (each 10 μ M) was added 30 min before (–0.5 hr) or at the indicated time points after the stimuli. MDL 12,330A (10 μ M) was added 30 min before (–0.5 hr), simultaneously (0 hr), or 10 min after the stimuli (0.17 hr). T cells were cultured with medium alone in parallel. Data are presented as percent inhibition calculated by the equation shown in "Materials and methods." The amount of IL-13 without inhibitors was as follows: 15 \pm 4 pg/mL in medium alone, 1315 \pm 148 pg/mL in PHA alone, and 1620 \pm 216 pg/mL in anti-CD3/CD28 alone (mean \pm SEM, N = 5).

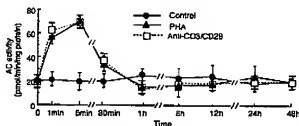


Fig. 3. Kinetics of AC activity. T cells from five different donors (two men and three women) were cultured with PHA (10 μ g/mL) or anti-CD3/CD28 (each 0.1 μ g/mL) in GAM-coated wells. T cells were cultured with medium alone in parallel. The AC activity was measured at the indicated time points after the stimuli. Values are means \pm SEM (N = 5).

PDE7 [25]. The increase of PDE1-, 2-, 3-, and 4-independent PDE activity was blocked by actinomycin D and cycloheximide (Fig. 5C), indicating the requirement of *de novo* protein synthesis. The increase of PDE1-, 2-, 3-, and 4-independent PDE activity was not blocked by H-89, indicating that protein kinase A may not be involved in the increase.

We then analyzed PDE1, PDE4, and PDE7 mRNA levels in PHA- or anti-CD3/CD28-stimulated T cells. PDE1B mRNA expression, undetectable in resting T cells, was induced by PHA or anti-CD3/CD28 after 30 min of lag time, and the expression was detected over a 2- to 24-hr period (Fig. 6). The PDE1B mRNA expression was not blocked by H-89, suggesting that protein kinase A may not be involved in the expression. PDE4A mRNA expression was detected in resting T cells, and the mRNA level was not

altered by either PHA or anti-CD3/CD28; the intensity ratio of PDE4A/ β -actin RT-PCR products in PHA- or anti-CD3/CD28-stimulated T cells was 94-104% of that in control T cells over a 0.5- to 24-hr period. The PDE4B and 4D mRNA levels were not increased by PHA or anti-CD3/CD28, either. PDE4C mRNA expression was not detected in any of control, PHA- or anti-CD3/CD28-stimulated T cells. Thus, PHA and anti-CD3/CD28 promoted PDE1 mRNA expression in T cells but not that of PDE4. PDE7 mRNA expression was detected in resting T cells, and the mRNA level was enhanced by anti-CD3/CD28 at 4 hr; the intensity ratio of PDE7/ β -actin RT-PCR products in anti-CD3/CD28-stimulated T cells was 3-fold of that in control T cells. The enhancement of PDE7 mRNA expression was specifically inhibited by PDE7 AS-O, but not by PDE7 NS-O. When the RT-PCR reaction was performed without reverse transcriptase, no detectable bands were obtained on agarose gels with the primer pair for PDE4A (Fig. 6) or the other pairs (data not shown), indicating that the bands seen for amplified cDNA were not due to the contamination of genomic DNA. To clarify the roles of AC and PDE isozymes in the regulation of the cAMP level, we examined the kinetics of the level of cAMP after PHA or anti-CD3/CD28 stimulation.

3.4. Kinetics of the intracellular cAMP level in PHA- or anti-CD3/CD28-stimulated T cells

As shown in Fig. 7, the cAMP level of T cells increased 2.1- or 2.2-fold above the basal level at 5 min after PHA or

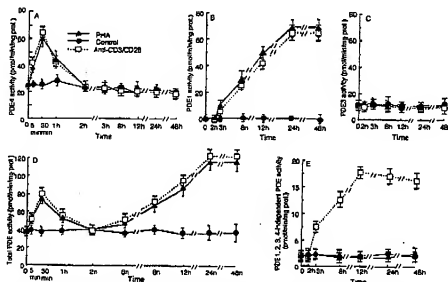


Fig. 4. Kinetics of total and isozyme-specific PDE activities. T cells from five different donors (two men and three women) were cultured with PHA (10 μ g/mL) or anti-CD3/CD28 (each 0.1 μ g/mL) in GAM-coated wells. T cells were cultured with medium alone in parallel. PDE1 (A), PDE3 (B), PDE4 (C), total PDE activity (D), and PDE1-, 2-, 3-, and 4-independent PDE activity (E) were measured at the indicated time points as described in "Materials and methods." Values are means \pm SEM (N = 5).

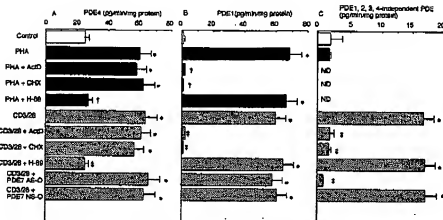


Fig. 5. Effects of various agents on PHA- or anti-CD3/CD28-induced increases of PDE4 (A), PDE1 (B), and PDE1-2, 3, 4-independent PDE (C) activities. T cells from five different donors (two men and three women) were cultured for 30 min (A), 24 hr (B), or 12 hr (C) with PHA (10 μ g/mL) or anti-CD3/CD28 (each 0.1 μ g/mL) in GAM-coated wells in the presence or absence of actinomycin D (AcD, 10 μ g/mL), cycloheximide (CHX, 10 μ g/mL), H-89 (0.5 μ M), PDE7 AS-O (20 μ M), or PDE7 NS-O (20 μ M). T cells were cultured with medium alone in parallel. Cells were lysed, and the activities of PDE subtypes were measured as described in "Materials and methods." Values are means \pm SEM (N = 5). ND, not done. Key: (*) $P < 0.05$ vs the values of control T cells with medium alone, (†) $P < 0.05$ vs the values of T cells with PHA alone, and (‡) $P < 0.05$ vs the values of anti-CD3/CD28 alone.

anti-CD3/CD28, respectively, then returned to the basal level at 2 hr, and continued to decrease further up to 24 hr, when the cAMP amount in PHA- or anti-CD3/CD28-stimulated cells was 25 or 20% of the basal level, respectively. The early increase of cAMP was consistent with the early activation of AC (Fig. 3), and was inhibited concentration-dependently by MDL 12,330A (Fig. 8); the IC_{50} values of MDL 12,330A for the inhibition of cAMP increase were 0.09 or 0.15 μ M in PHA- or anti-CD3/CD28-stimulated T cells, respectively, which was comparable to the IC_{50} values for the inhibition of IL-13 production (Fig. 1). These results indicate that the inhibition of IL-13 production by MDL 12,330A may be mediated by the inhibition of an early cAMP signal. Rolipram blocked the recovery from the elevated cAMP level for the first 2 hr after either stimulus, which is indicative of the transient activation of PDE4 in this phase (Fig. 4A). 8-Methoxymethyl-IBMX did not affect the kinetics of the cAMP level for the first 2 hr after either stimulus; however, it blocked the decrease of cAMP over a 2- to 48-hr period, which is consistent with the continuous activation of PDE1 in this phase (Fig. 4B). When both 8-methoxymethyl-IBMX and rolipram were added, the cAMP level remained elevated over a 48-hr period. These results suggest that PHA or anti-CD3/CD28 may initially (≤ 5 min) activate AC in T cells and increase cAMP. Thereafter, PDE4 and PDE1 may be successively activated and prevent prolonged cAMP accumulation; PDE4 may mainly reverse the early cAMP increase during the first 2 hr, while PDE1 may further reduce the cAMP level in the later phase. Both the time-dependent increase and decrease of cAMP may be required for IL-13 production.

4. Discussion

PHA or anti-CD3/CD28 transiently activated AC and generated cAMP signal in the early phase (≤ 5 min). PHA binds to the TCR/CD3 complex and CD2 on the T cell surface [26,27]. Although TCR/CD3 and CD2 do not directly couple to AC, PHA binding to these molecules triggers the activation of phospholipase C [28-30], and the phospholipase C-mediated signals activate AC [29]. The activated phospholipase C generates inositol 1,4,5-trisphosphate and diacylglycerol; the former induces intracellular Ca^{2+} mobilization and the latter activates protein kinase C [29]. It has been reported that Ca^{2+} forms a complex with cytosolic calmodulin and the latter activates protein kinase C also activates AC by phosphorylation [31]. Thus, PHA may activate AC indirectly via the TCR/CD3- and CD2-mediated phospholipase C pathway [32]. Since the stimulation of CD28 also triggers the activation of phospholipase C [33], CD3 and CD28 cross-linking may lead to AC activation via phospholipase C in a manner mimicking PHA. PHA or anti-CD3/CD28 increased the T cell cAMP level about 2-fold above the background, which was a smaller effect than that by typical AC stimulators; 10 μ M prostaglandin E_2 or 100 μ M forskolin increased the T cell cAMP level 18- to 20-fold above the background [9,18]. However, PHA- or anti-CD3/CD28-induced cAMP signal was necessary for IL-13 production and, thus, may be at least one of the triggering signals for IL-13 induction. To date, the involvement of AC in IL-13 production has not been studied precisely although previous papers suggest that cAMP may be the initial signal for DNA synthesis in PHA-

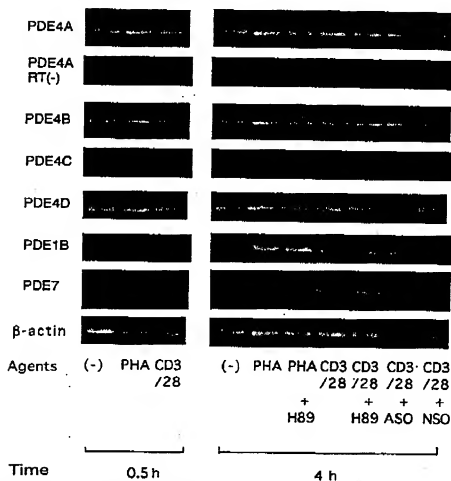


Fig. 6. RT-PCR analysis of PDE1, PDE4, and PDE7 mRNA expression. T cells from one healthy woman were cultured for 0.5 or 4 h with PHA (10 μ g/mL) or with anti-CD3/CD28 (each 0.1 μ g/mL) in GAM-coated wells in the presence or absence of H-89 (0.5 μ M), PDE7 AS-O (20 μ M), or PDE7 NS-O (20 μ M). RT-PCR was performed as described in "Materials and methods." Negative controls shown are the RT-PCR products for PDE4A performed without reverse transcriptase in cDNA synthesis (RT (-)). Data are representative of five separate experiments using T cells from five different donors (two men and three women).

stimulated T cells [34,35]. Since cAMP activates protein kinase A, this kinase may promote the early step of IL-13 production. One possible mechanism is that cAMP may induce the synthesis and/or activity of a certain transcription factor(s) crucial for IL-13 transcription. One candidate is AP-2 since an AP-2 binding site is present in the 5'-flanking region of IL-13 genes [36]. It has been reported that cAMP induces AP-2 transcription via protein kinase A in neuro-ectodermal cells [37,38]. It has also been suggested that cAMP may promote the interaction of AP-2 with another protein(s) to form more potent transcriptional complexes [39], or that cAMP may release an inhibitory protein(s), which may be constitutively associated with AP-2 and thus prevent the binding of AP-2 to DNA [40].

The PDE1 and 4 activities in T cells increased following AC activation, and also were required for IL-13 production.

PDE1 and 4 inhibitors prolonged cAMP accumulation, which appeared to suppress IL-13 production, thus suggesting that cAMP may inhibit the later events in IL-13 production. One possible mechanism is that cAMP may reduce the stability of IL-13 mRNA, which was also seen on IL-2 [41] or IL-4 mRNA in a previous study [10]. PDE1 and 4 may suppress cAMP accumulation and thus prevent the putative inhibitory effect of cAMP in the later phase.

The transient activation of PDE4 did not require *de novo* protein synthesis but involved protein kinase A. These findings indicate that protein kinase A, which is activated by the early cAMP signal, may activate PDE4 directly; it has been reported that protein kinase A phosphorylates PDE4D3 at serine 54, which induces rapid and reversible activation of PDE4D3 [42]. In the present study, the transiently activated PDE4 reversed the initial cAMP increase by hydrolysis,

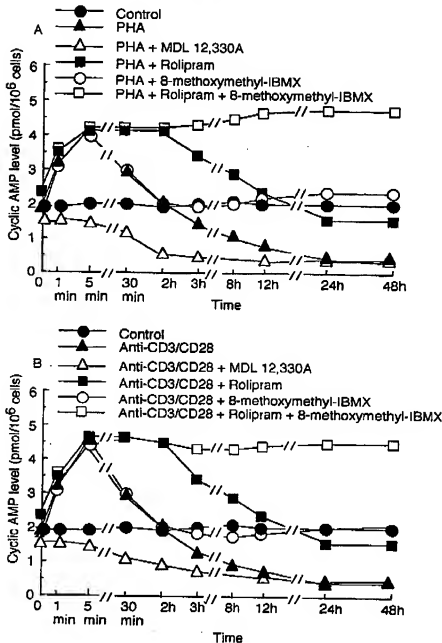


Fig. 7. Kinetics of the intracellular cAMP level. T cells from one healthy woman were preincubated for 30 min with medium alone or with medium containing MDL 12,330A (10 μ M), or rolipram and/or 8-methoxymethyl-IBMX (each 10 μ M) in GAM-coated wells; then PHA (final concentration of 10 μ g/mL) (A) or anti-CD3/CD28 (each 0.1 μ g/mL) (B) was added. T cells were cultured with medium alone in parallel. The intracellular cAMP level was analyzed at the indicated time points after the stimuli. The mean of triplicate cultures is shown; the SDs were <10% of the means. The data are representative of five separate experiments using T cells from five different donors (two men and three women).

indicating a negative feedback control of the level of cAMP. Recent studies have also reported that PDE4 transcription is promoted by a prolonged and excessive cAMP signal, 8-bromo-cAMP or forskolin-enhanced PDE4 transcription

in T cells [18,43], indicating the presence of cAMP-inducible sites in the PDE4D promoter. In the present study, however, neither PHA nor anti-CD3/CD28 enhanced the level of PDE4D mRNA. This is possibly because the PHA-

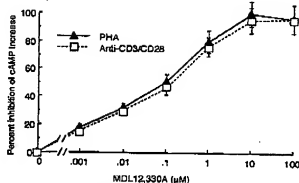


Fig. 8. Concentration-dependency for the inhibitory effects of MDL 12,330A on PHA- or anti-CD3/CD28-induced cAMP increases. T cells from five different donors (two men and three women) were preincubated for 30 min with medium alone or with medium containing MDL 12,330A at the indicated concentrations in GAM-coated wells; then PHA (final concentration of 10 μg/mL) or anti-CD3/CD28 (each 0.1 μg/mL) was added. T cells were cultured with medium alone in parallel. The intracellular cAMP level was analyzed at 5 min after the stimuli. Data are presented as percent inhibition calculated by the following equation: (cAMP amount with stimulus alone - cAMP amount with stimulus plus inhibitor) ÷ (cAMP amount with stimulus alone - cAMP amount with medium alone) × 100 (%). Values are means ± SEM (N = 5). The cAMP amount without MDL 12,330A was as follows: 2.1 ± 0.3 pmol/10⁶ cells in medium alone, 4.3 ± 0.6 pmol/10⁶ cells in PHA alone, and 4.6 ± 0.8 pmol/10⁶ cells in anti-CD3/CD28 alone (mean ± SEM, N = 5).

or anti-CD3/CD28-induced cAMP signal was transient, and thus may be insufficient for the transcriptional induction.

The enzymatic activity and mRNA expression of PDE1, undetectable in resting T cells, were induced by PHA or anti-CD3/CD28. Protein kinase A was not involved in the PDE1B mRNA expression. Previous studies supported the hypothesis that PHA induces PDE1 mRNA expression and enzymatic activity [44,45], although PDE1 induction by anti-CD3/CD28 has not been reported. Our present results indicate that PDE1B transcription may possibly be induced by certain protein kinase A-independent signals mediated commonly by PHA and anti-CD3/CD28; one candidate is protein kinase C since both PHA and anti-CD3/CD28 activate protein kinase C via phospholipase C in T cells [28]. Spence *et al.* [19] also reported that the protein kinase C activator phorbol 12-myristate 13-acetate induces PDE1 mRNA expression in Chinese hamster ovary cells.

In this study, PDE7 mRNA expression in T cells was enhanced by anti-CD3/CD28 but not by PHA, which is consistent with results in recent studies [24,46]. It is thus indicated that PDE7 mRNA expression may be mediated via certain CD28-triggered signals that may not be induced by CD2 or CD3, such as acidic sphingomyelinase [47]. Anti-CD3/CD28 increased PDE1-, 2-, 3-, and 4-independent PDE activity while PHA did not. The increase of this activity was blocked by PDE7 AS-O, indicating the contribution of PDE7. However, we cannot verify that the PDE1-, 2-, 3-, and 4-independent PDE activity is authentically that

of PDE7 due to the lack of a PDE7-selective inhibitor. Besides, we cannot currently examine if PDE7 AS-O can reliably abrogate PDE7 protein expression in anti-CD3/CD28-activated T cells by Western blot or similar experiments since PDE7-specific antibody is not available. In our preliminary studies, PDE7 AS-O slightly reduced IL-13 production in anti-CD3/CD28-activated T cells (approximately 15%), which was not statistically significant (data not shown). Thus, it still seems premature to conclude that PDE7 may regulate IL-13 production in anti-CD3/CD28-activated T cells. The possible role of PDE7 should be elucidated in future studies. The PDE1-, 2-, 3-, and 4-independent PDE activity may also consist of other isozymes different from PDE7, such as cAMP-specific IBMX-insensitive PDE (PDE8) [48], although the activity of these isozymes may be low. Since specific inhibitors for these isozymes are not currently available, we cannot examine their activities or those of PDE7 correctly. However, it is possible that these minor PDE isozymes may be related to IL-13 production in T cells. This possibility should be investigated further with developing selective inhibitors for the isozymes.

AC, PDE1, and PDE4 inhibitors suppressed IL-13 production. Since IL-13 is closely related to the development of atopic dermatitis, asthma, or allergic rhinitis [2,3], these agents can be used therapeutically for these allergic diseases. Patients with atopic dermatitis are particularly associated with increased PDE activity [49], which may be related to the increased IL-13 production in these patients. Therefore, it is expected that a PDE1 and/or PDE4 inhibitor may prevent the development of atopic dermatitis via the inhibition of IL-13 production. We are now studying the effects of these agents on IL-13 production by the T cells of these patients.

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toward SDF-1 and efficient engraftment by converted CD34⁺CD38^{low}CXCR4⁺ cells, properties that were similar to those of the original migrating fraction (M) (Fig. 4A).

Self-renewal of stem cells can only be determined by their ability to also repopulate secondary transplanted recipients with high numbers of both myeloid and lymphoid cells. Consistent with previous studies, secondary transplanted mice that received untreated human cells showed little engraftment (Fig. 4B, panel a) (23). Human interleukin-6 (IL-6) synergizing with SCF induced high levels of CXCR4 expression on CD34⁺ cord blood cells (Fig. 4C). Incubation of bone marrow cells from primary transplanted mice with SCF and IL-6 for 48 hours resulted in up-regulation of surface CXCR4 expression (Fig. 4B, panel c) and increased migration of human progenitor cells to SDF-1 *in vitro* (Fig. 4B, panel d). Transplantation of similar numbers of human cells from the bone marrow of primary transplanted mice after treatment with these cytokines resulted in higher engraftment levels in secondary transplanted mice compared with mice transplanted with untreated cells (Fig. 4B, panel b versus panel a). Thus, by up-regulating surface CXCR4 expression on primitive cells, the population of self-renewing CD34⁺CD38^{low}SRC stem cells could be increased.

Our data provide evidence that CXCR4-dependent migration to SDF-1 is essential for human stem cell function in NOD/SCID mice. We characterized SRCs further as CD34⁺CD38^{low}CXCR4⁺ stem cells and showed that CD34⁺CD38^{low}CXCR4⁺ stem cells can be converted into functional CXCR4⁺ stem cells by cytokine treatment. This suggests that migration to SDF-1 is associated with localization of stem cells in the bone marrow, permitting differentiating cells with reduced migration levels to exit into the blood circulation. In conclusion, our findings define human CD38^{low}CXCR4⁺ cells as stem cells endowed with migration and repopulation potential and provide insights into human stem cell biology.

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23. Human cells were obtained after informed consent according to procedures approved by the Weizmann Committee. In all experiments, samples of the same initial cell pool were compared. Differences in the results are due to the different CD34⁺ cell sources (cord blood, bone marrow, and mobilized peripheral blood). CD34⁺ enrichment, flow cytometry, and fluorescence-activated cell sorting (FACS) were performed as previously described (5, 6). 501-1 (125 ng/ml, R&D Systems) transmigration assays were done as previously described (13) with 2×10^4 CD34⁺ cells. Percentages in the results represent percent of initial 2×10^4 cells in the migrating and nonmigrating cell fractions. The sources for the reagents are as follows: PMA (100 ng/ml) was purchased from Sigma; stem cell factor (SCF) and IL-6 (50 ng/ml) from R&D Systems; and antibodies to CXCR4 from Pharmingen (12G5 monoclonal antibody [immunoglobulin G2a (IgG2a)] or R&D Systems [MAB171 monoclonal antibody (IgG2a)] 150 µg per 2 × 10⁴ cells). CXCR4 expression was always analyzed by double staining with anti-CD34. Polyclonal anti-SDF-1 (10 µg per mouse, R&D Systems) was injected intravenously with the cells (2×10^4 cells per mouse) and 24 hours later injected again intraperitoneally. Control cells were incubated with anti-CD34 (IGT, Boston Biosciences, 10 µg per 2 × 10⁴ cells). Human lymphoid and myeloid cells were immunostained with anti-CD45 (Immunocyt Quality Products, Criegee, Netherlands), anti-CD18, and anti-CD36 (Coulter). Natural killer cells differentiated into mature CD36⁺ cells after incubation with human SCF (100 ng/ml) and human IL-15 (100 ng/ml, R&D Systems) for 10 days. NOD/SCID and NOD.SID β₂-microglobulin knockout (20) mice were bred and maintained under defined conditions in immunodeficient cages and transferred by injection into the tail vein after sublethal (575R) irradiation according to established protocols (5, 6) approved by the Weizmann animal ethics committee. Southern (DNA) blot analysis with a human-specific α-satellite probe and human-specific progenitor assays were done as previously described (5, 6). Percent engraftment always indicated the percent of either human DNA or of human CD45 cells in the mouse bone marrow. The levels of engraftment were dependent on the injected cell dose, the duration of the experiment, and the source of human CD34⁺ cells. Cells were cultured either in serum-free media as previously described (6) or in media supplemented with 10% fetal calf serum.

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CD3- and CD28-Dependent Induction of PDE7 Required for T Cell Activation

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Costimulation of both the CD3 and CD28 receptors is essential for T cell activation. Induction of adenosine 3',5'-monophosphate (cAMP)-specific phosphodiesterase-7 (PDE7) was found to be a consequence of such costimulation. Increased PDE7 in T cells correlated with decreased cAMP, increased interleukin-2 expression, and increased proliferation. Selectively reducing PDE7 expression with a PDE7 antisense oligonucleotide inhibited T cell proliferation; inhibition was reversed by blocking the cAMP signaling pathways that operate through cAMP-dependent protein kinase (PKA). Thus, PDE7 induction and consequent suppression of PKA activity is required for T cell activation, and inhibition of PDE7 could be an approach to treating T cell-dependent disorders.

Activation of peripheral T cells *in vivo* by an antigen-presenting cell is a result of the engagement of both the T cell receptor-CD3 complex (TCR-CD3) and the CD28 costimu-

latory receptor. When both receptors are occupied by their appropriate ligands, T cells are stimulated to proliferate and produce interleukin-2 (IL-2), whereas occupation of the T cell receptor alone favors T cell anergy or apoptosis (1). Occupation of the CD28 receptor alone appears to have no obvious effect on T cells; nevertheless, CD28 costimulation is required for full activation of CD4 T helper cells, if not all T cells (2). Why is CD28 costimulation required for T cell activation? One possible reason has been suggested by

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the observation that ligation of TCR-CD3 initiates not only stimulatory signal transduction pathways, such as the MAP kinase (mitogen-activated protein kinase)-dependent signaling pathways (3) and the NFAT (nuclear factor of activated T cells)-dependent signaling pathways (4), but also inhibitory signal transduction pathways such as the PKA (the cAMP-dependent protein kinase)-dependent signaling pathway (5). PKA can inhibit proliferation in many cell types, including fibroblasts (Rat-1 and NIH 3T3 cells), smooth muscle cells, and adipocytes (6), as well as T cells (7). Phosphorylation of NFAT by PKA can abolish the translocation of NFAT from the cytoplasm to the nucleus (8); and phosphorylation of Raf kinase, again by PKA, can block the MAP kinase-dependent signaling pathway (9). Both the translocation of NFAT and the MAP kinase-dependent signal are essential for IL-2 gene expression (3, 4). It is plausible, therefore, that to become fully activated, T cells require additional TCR-independent signals to overcome PKA inhibition.

Because phosphodiesterases (PDEs) play a major role in down-regulation of PKA activity by hydrolyzing cAMP in many cell types (10), and PDE7 protein appears to be detected only in lymphocytes (11), we reasoned that PDE7 might play a role in regulating T cell activation. To test this hypothesis, we first analyzed Northern (RNA) blots to determine the tissue distribution of PDE7. Consistent with previous work demonstrating that PDE7 contributed up to 40% of total PDE activity in several T cell lines (1), PDE7A1, one of the two RNA splicing variants of PDE7, is predominantly expressed in human lymphoid tissues (Fig. 1B). However, the PDE7 protein was barely detectable in isolated peripheral T cells (Fig. 2A), but PDE7 activity was increased by costimulation of T cells with antibodies to CD3 and CD28 (Fig. 2B); whereas the activity of PDE4, the other major isoform of cAMP-specific PDE in T cells, remained unchanged (12). Very high doses of antibody to CD3 (anti-CD3) or antibody to CD28 (anti-CD28) alone could induce PDE7 (12). However, at lower doses that are more likely to mimic the normal physiological state (0.2 ng of anti-CD3 per milliliter and 0.2 μ g of anti-CD28 per milliliter), only the combination of the two antibodies increased the amount of PDE7 protein (Fig. 2A) and its activity (Fig. 2B), decreased cAMP levels (Fig. 2C), increased IL-2 expression (Fig. 2D), and promoted proliferation (Fig. 2E), in isolated peripheral T cells.

To investigate whether induction of PDE7 was not merely correlated with but essential for T cell proliferation, we tested the effect of blocking PDE7 expression on T cell proliferation as well as on IL-2 production. Because no PDE7-specific inhibitor is yet available, we used PDE7 antisense oligonucleotides to block

PDE7 expression. Several nonoverlapping antisense oligonucleotides were tested. In addition, a series of control oligonucleotides that

shared the same nucleotide composition with their corresponding antisense oligonucleotide but had a reversed sequence order were also

Fig. 1. Northern blot analysis of PDE7A1 and PDE7A2. (A) PDE7A2 is expressed in heart and skeletal muscle, whereas PDE7A1 is expressed in the pancreas and the placenta. (B) PDE7A1 is predominantly expressed in lymphoid tissues. Northern blots of multiple human tissues [sample number 7760-1 in (A) and 7768-1 in (B)] (Clontech, Palo Alto, CA) were hybridized with a ³²P-labeled, 1.2-kb, Not I-Eco RI fragment of human PDE7 cDNA, which can hybridize with both PDE7A1 and PDE7A2. Express-hyb solution (Clontech) was used for both prehybridization and hybridization performed at 60°C. The ³²P-labeled probe, together with salmon sperm DNA (0.1 mg/ml), was added directly to the prehybridization solution for hybridization. Membranes were washed twice in 2× SSC [3 M sodium chloride and 0.3 M sodium citrate (pH 7.0)] and 0.1% SDS at room temperature for 10 min, then once for the mRNA quantity of the Northern blot. Relative molecular weights are indicated. 7A1 stands for the 4.2-kb mRNA of PDE7A1 (15) and 7A2 for the 3.8-kb mRNA of PDE7A2 (11, 15). 7A3? indicates a possible third splice variant, PDE7A3.

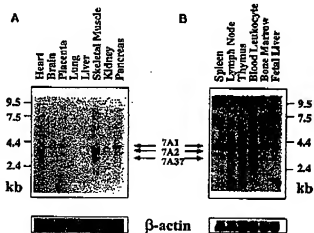
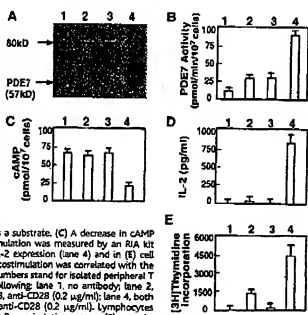
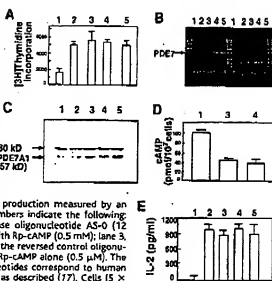


Fig. 2. Induction of PDE7 in peripheral T cells by anti-CD3 and anti-CD28 costimulation. (A) Protein immunoblot analysis of PDE7 protein. The 57-kD band (lane 4) is the predicted size of the PDE7A1 protein. The 80-kD band, which cross-reacts with PDE7 antibodies, is an unknown protein present in the particulate fraction and appears to have no PDE activity. (B) PDE activity in the immunoprecipitated protein of PDE7-specific antibodies was measured using 1 μ M cAMP as a substrate. (C) A decrease in cAMP levels (lane 4) upon costimulation was measured by an RIA kit (15). (D) The increase in IL-2 expression (lane 4) and in (E) cell proliferation (lane 4) after costimulation was correlated with the PDE7 induction. The lane numbers stand for isolated peripheral T cells incubated with the following: lane 1, no antibody; lane 2, anti-CD3 (0.2 ng/ml); lane 3, anti-CD28 (0.2 μ g/ml); lane 4, both anti-CD3 (0.2 ng/ml) and anti-CD28 (0.2 μ g/ml). Lymphocytes were isolated with a Ficoll-Paque isolation system (Pharmacia, Uppsala, Sweden). B cells were depleted with anti-CD19 conjugated Dynabeads M-450 according to the protocol provided (Dynal, Lake Success, New York). Isolated peripheral T cells were plated into a six-well plate precoated with goat antibodies to mouse IgG in RPMI 1640 containing 10% fetal calf serum (5 × 10⁶ cells per well). The cells were then stimulated with anti-CD3 alone, anti-CD28 alone, or a combination of both. Eight hours after incubation, the supernatant of the cell culture was collected for assay of IL-2 with an enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Woburn, Massachusetts), and the cells were harvested for protein immunoblot analysis, immunoprecipitation, radioimmunoassay of cAMP, and cell proliferation (16).



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Fig. 3. The effect of a PDE7 antisense oligonucleotide on proliferation and IL-2 production in HuT78 T cells. (A) PDE7 antisense oligonucleotide, AS-0, inhibits T cell proliferation. (B) PDE7 mRNA was reduced in the AS-0-treated cells analyzed by RT-PCR. (C) AS-0 reduced the 80-kD PDE7 protein band but did not affect the 80-kD band. (D) AS-0 increased cAMP levels in the cell. (E) AS-0 attenuated IL-2 production measured by an ELISA assay. The lane numbers indicate the following: lane 1, the PDE7 antisense oligonucleotide AS-0 (12 μ M); lane 2, AS-0 along with Rp-cAMP (0.5 mM); lane 3, RpH₂1640 media; lane 4, the reverse control oligonucleotide (12 μ M); lane 5, Rp-cAMP alone (0.5 μ M). The three antisense oligonucleotides correspond to human PDE7A1 cDNA sequences as described (17). Cells (5×10^5 per well) were treated with 12 μ M of PDE7 antisense oligonucleotides or with the reverse control oligonucleotides in RPMI 1640 media containing 10% fetal calf serum. To test the effect of PKA inhibitors on cell proliferation, 0.5 mM Rp-cAMP (BioLabs, La Jolla, CA) was added together with the oligonucleotides. Twenty-four hours after incubation, the cells were labeled with 1 μ Ci of [3 H]thymidine per well for 20 hours and then harvested for scintillation counting. For RT-PCR analysis, total RNA was isolated with a TRIzol RNA isolation kit (Gibco-BRL). cDNA was synthesized with the Superscript II reverse transcription system (Gibco-BRL). One-tenth the volume of the reverse-transcribed cDNA was amplified with PDE7- or PDE4-specific primers (17). The products were subjected to electrophoresis on 2% agarose gels.



[3H]Thymidine incorporation

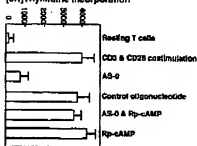


Fig. 4. Inhibition of primary CD4 T cell proliferation by PDE7 antisense oligonucleotides. Peripheral T cells were isolated with anti-CD4-conjugated Dynabeads M-450 according to the provided protocol (Dyna). The isolated CD4 T cells were activated by anti-CD3 (0.2 ng/ml) together with anti-CD28 (0.2 μ g/ml), with or without 12 μ M of PDE7 antisense AS-0 or the reverse control oligonucleotides, in a 96-well plate pre-coated with goat antibodies to mouse. Rp-cAMP (0.5 mM) was added along with the oligonucleotides in the defined wells. After 24 hours of incubation, [3 H]thymidine was added and the cells were incubated for another 24 hours. Cell proliferation was measured as described above.

tested. As shown in Fig. 3, 12 μ M of the AS-0 oligonucleotide inhibited cell proliferation by 70% (Fig. 3A, lane 1) and attenuated IL-2 production (Fig. 3E, lane 1) in HuT78 T cells, which constitutively express high levels of PDE7 activity (17); incubation with the other two antisense oligonucleotides resulted in 20% inhibition or less (12). None of the control oligonucleotides had any effect on T cell proliferation and IL-2 production at this concentration (Fig. 3A and E, lane 4).

To determine whether AS-0 treatment induced a decrease in PDE7 expression, we performed reverse transcription polymerase chain reaction (RT-PCR) analysis of PDE7 mRNA and protein immunoblot analysis of PDE7 protein from the HuT78 cells treated with antisense oligonucleotides or with the control oligonucleotides. Both the mRNA (Fig. 3B, lane 1) and the protein (Fig. 3C, lane 1) of PDE7 were decreased in the AS-0-treated cells but were unchanged in the cells treated with control oligonucleotide (Fig. 3B and C, lane 4). The inhibition by the antisense oligonucleotide was selective for PDE7, because the PDE4 mRNA remained unchanged in all the cells tested (Fig. 3B, right panel). cAMP concentration in the AS-0-treated cells was increased 2.5 times as compared with cells treated with the control oligonucleotides (Fig. 3D). Therefore, inhibition of T cell proliferation and IL-2 production by PDE7 antisense oligonucleotide ap-

peared to be mediated through the targeting of PDE7 gene expression and consequent increases in cAMP and PKA activity. If this were the case, one would predict that suppression of PKA activity should reverse the inhibition of proliferation and IL-2 production by the PDE7 antisense oligonucleotide. As shown in Fig. 3, Rp-cAMP (0.5 mM Rp-adenosine 3',5'-cyclic monophosphothioate), a PKA-specific inhibitor, completely reversed both the inhibition of cell proliferation (Fig. 3A, lane 2) and the attenuation of IL-2 production (Fig. 3E, lane 2) in HuT78 cells that had been caused by treatment with AS-0, whereas Rp-cAMP itself had no obvious effect on the cell (Fig. 3A and E, lane 5).

Conclusions based on studies with growth factor-independent T cell lines do not necessarily hold true in primary T cells. To confirm the role of PDE7 in T cell activation, we tested the effect of the PDE7 antisense oligonucleotide on isolated peripheral CD4 T cells that had been activated by anti-CD3 and anti-CD28. As expected, the PDE7 antisense oligonucleotide AS-0 inhibited T cell proliferation by 80%, whereas the reverse control oligonucleotide showed no inhibition (Fig. 4). The PKA inhibitor Rp-cAMP blocked the inhibition of proliferation in the PDE7 antisense AS-0-treated cells (Fig. 4). Therefore, induction of PDE7 is essential for T cell activation.

It is still not clear how PDE7 protein and

activity are increased upon activation of T cells. Easily detectable amounts of PDE7 mRNA were constitutively expressed in resting T cells (Fig. 1B). Therefore, the increase in PDE7 protein amount and activity may result from an up-regulation of protein translation or a decrease in degradation.

It has been reported that PDE4 inhibitors can also be effective in blocking T cell proliferation and that PDE3-inhibitors potentiate this blocking effect (13). None of the inhibitors used in those studies are thought to inhibit PDE7, thus raising the question of how all three of these PDE isozymes could be involved in T cell proliferation. This issue remains unresolved, but it is perhaps worth noting that in other cell types, different PDEs have been shown to be differentially localized within the cell (10). As a result of this localization, the regulation (14) and function of different PDEs may vary accordingly (10). It is also possible that changes in cAMP modulated by different PDE isozymes work at different phases of the cell cycle. In either of these cases, selective inhibitors of specific PDEs might be expected to inhibit the overall process of cell proliferation.

Our data provide evidence for a mechanism of CD28-dependent costimulation during T cell activation. The data also suggest that PDE7 may be a good target for selective therapeutic modulation of T cell responsiveness.

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Requirement of Cdk2-Cyclin E Activity for Repeated Centrosome Reproduction in *Xenopus* Egg Extracts

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The abnormally high number of centrosomes found in many human tumor cells can lead directly to aneuploidy and genomic instability through the formation of multipolar mitotic spindles. To facilitate investigation of the mechanisms that control centrosome reproduction, a frog egg extract arrested in S phase of the cell cycle that supported repeated assembly of daughter centrosomes was developed. Multiple rounds of centrosome reproduction were blocked by selective inactivation of cyclin-dependent kinase 2-cyclin E (Cdk2-E) and were restored by addition of purified Cdk2-E. Confocal immunocytochemistry revealed that cyclin E was localized at the centrosome. These results demonstrate that Cdk2-E activity is required for centrosome duplication during S phase and suggest a mechanism that could coordinate centrosome reproduction with cycles of DNA synthesis and mitosis.

In animal cells, the interphase centrosomes reproduce or duplicate only once per cell cycle, thereby ensuring a strictly bipolar mitotic spindle axis (1). Because there is no cell cycle checkpoint that monitors the number of spindle poles (2), uncontrolled duplication of the centrosome can contribute to genomic instability through the formation of multipolar mitotic spindles. Indeed, many human tumor cells, including those lacking the tumor suppressor protein p53 (3), have abnormally high numbers of centrosomes (4).

Studies of sea urchin and *Xenopus* embryos and clam oocyte lysates have revealed that the centrosome cycle can be regulated solely by cytoplasmic mechanisms (5–8). The repeated duplication of the centrosome proceeds in the complete absence of either a nucleus (7) or protein synthesis (8). In theory, the cyclical rise and fall in the activity of one or more cyclin-dependent kinases (Cdks) could be the cytoplasmic mechanism that coordinates centrosome reproduction with cell cycle progression. However, the fact that centrosomes repeatedly duplicate in the complete absence of protein synthesis indicates that the activities of those Cdks that are dependent on the translation of their cyclin subunits during each cell cycle (that is, Cdk1-cyclin A or -cyclin B or both) do not regulate centrosome reproduction or assembly (8). Nevertheless,

Cdk2-cyclin E (Cdk2-E) remains a potential candidate to control centrosome duplication and coordinate it with nuclear events during the cell cycle (6, 9, 10). Cdk2-E activity drives the transition from G₁ to S phase in somatic cells (11), which is the time during the cell cycle when daughter centrosome assembly is thought to begin (12). Importantly, in early *Xenopus* embryos, Cdk2-E activity is not dependent on the synthesis and degradation of the cyclin E subunit, as the amount of cyclin E remains constant until the mid-blastula transition (MBT) (13).

To investigate whether Cdk2-E activity regulates centrosome duplication, we developed an S phase-arrested *Xenopus* egg extract that supports repeated centrosome reproduction in vitro. We used an S phase extract because centrosomes will undergo multiple rounds of duplication during S phase arrest in both zygotes and somatic cells (6, 14, 15). Unlike cycling oocytes, Cdk2-E activity can be inhibited in S phase-arrested extracts without the concern that this inhibition will block cell cycle progression at a point before centrosomes are normally scheduled to reproduce. To make these extracts, we prepared a cycling *Xenopus* egg extract (16, 17) and then added aphidicolin, an inhibitor of α -DNA polymerase (18), and desteminated *Xenopus* sperm nuclei (19). Histone H1 kinase activity in control extracts cycled at least twice with a cell cycle time of ~50 min; in contrast, H1 activity in aphidicolin-treated extracts remained at a constant, low amount for 6 hours (20, 21). Time-lapse videomicroscopy of aphidicolin-treated extracts revealed that nuclear envelope breakdown did not occur during the 6-hour experiment (20). Thus,

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14. For the protein immunoblot analysis, 20 μ g of total protein was loaded in each lane and separated with 8% SDS-polyacrylamide gel electrophoresis. Affinity-purified antibody 6B58 to p107, directed with phosphate-buffered saline (PBS) containing 5% nonfat milk, was used for protein immunoblot analysis. Specific protein bands were revealed by the ECL system (Pierce & Warriner). For the immunoprecipitation and PDI assay, 10 million isolated peripheral T cells were homogenized with 1 ml of 40 mM Tris-HCl buffer (homogenizing buffer) containing 1 mM EDTA, 5 mM dithiothreitol, 1 μ M pepstatin (Sigma), and 10 μ M leupeptin (Sigma). After centrifugation for 20 min at 14,000 rpm (Microcentrifuge), the supernatant was saved and was incubated overnight at 4°C with 1 μ g of affinity-purified antibody 6B58. Protein A-Sepharose (200 μ l of a 5% suspension) was then added, and the mixture was incubated for 3 hours at 4°C. The protein A-bound proteins were washed twice with PBS and resuspended with the homogenizing buffer for measurement of PDI activity, using 1 μ M cAMP as a substrate. For the radioimmunoassay of GMP, cells were homogenized in 5% trichloroacetic acid according to the protocol provided with the radioimmunoassay system (NEN, Boston, MA). To measure proliferation, cells (10⁵ cells per well) were plated in a 96-well plate precoated with goat antibodies to 3 H-TdR and were incubated with anti-Cdk2 or anti-Cdk2-E for 6 hours. A microtiter of 0.1 μ M thymidine was then added per well. Sixteen hours later, cells were harvested for scintillation counting.
15. The three PDE7 antisense oligonucleotides were as follows: from position 1 to 24 (AS-5): 5'-CGGACGCTCTACACACATCTCAT; from position 700 to 728 (AS-708): 5'-CAGCTCAGGCTGACCTAAGC; and from position 887 to 899 (AS-887): 5'-CGGACGCTCTACAGTAAGCTCT. For RT-PCR analysis, PDE7-specific primer pairs were as follows: 5'-CATTTTGTGACCATCTGCGAGG-3', 5'-GACGCTTCGCGGCTACTCTATGCA-3'. PDE4A-specific primer pairs were as follows: AACAGCAACAGAAATATCAATCG and TATACAGACACCAATCTCTC.
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Potential role of phosphodiesterase 7 in human T cell function: comparative effects of two phosphodiesterase inhibitors

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SUMMARY

Even though the existence of phosphodiesterase (PDE) 7 in T cells has been proved, the lack of a selective PDE7 inhibitor has confounded an accurate assessment of PDE7 function in such cells. In order to elucidate the role of PDE7 in human T cell function, the effects of two PDE inhibitors on PDE7A activity, cytokine synthesis, proliferation and CD25 expression of human peripheral blood mononuclear cells (PBMC) were determined. Recombinant human PDE7A was obtained and subjected to cyclic AMP-hydrolysis assay. PBMC of *Dermatophagoides farinae* mite extract (Df)-sensitive donors were stimulated with the relevant antigen or an anti-CD3 monoclonal antibody (MoAb). PBMC produced IL-5 and proliferated in response to stimulation with Df, while stimulation with anti-CD3 MoAb induced CD25 expression and messenger RNA (mRNA) synthesis of IL-2, IL-4 and IL-5 in peripheral T cells. A PDE inhibitor, T-2585, which suppressed PDE4 isoenzyme with high potency ($IC_{50} = 0.00013 \mu M$) and PDE7A with low potency ($IC_{50} = 1.7 \mu M$) inhibited cytokine synthesis, proliferation and CD25 expression in the dose range at which the drug suppressed PDE7A activity. A potent selective inhibitor of PDE4 ($IC_{50} = 0.00031 \mu M$), RP 73401, which did not effectively suppress PDE7A ($IC_{50} > 10 \mu M$), inhibited the Df- and anti-CD3 MoAb-stimulated responses only weakly, even at $10 \mu M$. PDE7 may play a critical role in the regulation of human T cell function, and thereby selective PDE7 inhibitors have the potential to be used to treat immunological and inflammatory disorders.

Keywords cyclic AMP cytokine phosphodiesterase T cell

INTRODUCTION

Cyclic AMP (cAMP) has been recognized as an important second messenger regulating immune and inflammatory responses. Agents with the ability to elevate intracellular cAMP levels have been demonstrated to possess immunosuppressive and anti-inflammatory properties [1,2]. These effects are caused in part by the inhibition of various T cell functions including proliferation [3], cytokine production [4-6] and expression of activation markers on the cell surface [7].

One mechanism by which cAMP may be elevated within cells is by inhibition of phosphodiesterase (PDE). PDE comprises a family of enzymes, currently known to exist in at least 11 different isoenzyme forms which are characterized by a variety of properties, including their sensitivity to different inhibitors [8-12]. With regard to T cell function, the existence of three distinct cAMP-PDE isoenzymes, PDE3, 4 and 7, has been implicated [13-16]. However, earlier studies have not clarified the comparative roles of these isoenzymes in the degradation of intracellular

cAMP in human T cells. PDE4 is one of the most convincing candidates for this role, as inhibition of various T cell functions by 'so-called' selective PDE4 inhibitors has been documented [14,16-19]. Nevertheless, several earlier studies are in apparent contradiction with the idea of a principle role for PDE4, as the potency of PDE4 inhibitors to suppress cytokine production and proliferation of human peripheral T cells was not correlated with their ability to suppress PDE4 activity [19-23]. Regarding PDE3, few reports support the contribution of PDE3 in the regulation of T cell function, and a lack of effect of various PDE3 inhibitors on T cell activation has been documented [16-19,22,23]. The existence of a rolipram-insensitive cAMP-PDE type enzyme in T cells was described first by Ichimura and Kase [13] and thereafter it was identified as PDE7A by Bloom and Bravo [24]. However, the role of PDE7 in T cell function is still unclear, as a selective PDE7 inhibitor had not been established.

We have generated a new PDE inhibitor, T-2585 (2-[4-[2,3-bis(hydroxymethyl)-6,7-diethoxy-1-naphthalenyl]-2-pyridinyl]-4-(3-pyridinyl)-1(2H)-phthalazine) [25]. This agent displays potent inhibitory activity against PDE4 ($IC_{50} = 0.00013 \mu M$) and 14 000-fold greater selectivity compared with PDE3 [25]. Another PDE4 inhibitor, RP 73401 (3-cyclopentylethoxy-N-(2,6-dichloro-4-pyridyl)-4-methoxybenzamide), has been evaluated to have

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similar potency of PDE4 suppression ($IC_{50} = 0.00031 \mu M$) to T-2585 [25,26]. RP 73401 failed to suppress the activity of other PDE isoenzymes even at $10 \mu M$ [25-27].

In this study, we obtained an interesting result in that T-2585 suppressed PDE7A activity with an IC_{50} of $1.7 \mu M$, whereas RP 73401 suppressed it only weakly at $10 \mu M$. This finding provides a useful insight into the significance of PDE7 in T cell function, since it is possible to predict the potential role of PDE7 by comparing the effects of the two PDE inhibitors. In this study, therefore, the effects of T-2585 and RP 73401 on various T cell functions were characterized using human peripheral blood mononuclear cells (PBMC).

MATERIALS AND METHODS

Materials

T-2585 and RP 73401 were synthesized by the Discovery Research Laboratory, Tanabe Seiyaku Co. (Osaka, Japan). *Derma-maphagoides farinae* mite extract (*Df*; Torii Pharmaceutical Co., Tokyo, Japan), anti-CD3 monoclonal antibody (MoAb; Orihu, Raritan, NJ, USA), purified rat antiserum/human IL-5 monoclonal antibody (Pharmingen, San Diego, CA, USA), biotinylated rat antihuman IL-5 monoclonal antibody (Pharmingen), Cell Titer 96™ Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA), ISOGEN (Nippongene, Tokyo, Japan), murine Moloney leukaemia virus reverse transcriptase (Perkin-Elmer Cetus, Norwalk, CT, USA), GeneAmp® DNA polymerase (Perkin-Elmer Cetus), RT-PCR Amplifier Sets (Clontech, Palo Alto, CA, USA), fluorescein isocyanate (FITC)-labelled anti-CD25 antibody (Becton Dickinson, Franklin Lakes, NJ, USA), phycoerythrin (PE)-labelled anti-CD3 antibody (Becton Dickinson), Ficol-Paque (Pharmacia, Uppsala, Sweden) and AIM-V medium (Gibco BRL, Gaithersburg, MD, USA) were used. All other reagents were obtained from Sigma (St Louis, MO, USA).

Expression of recombinant PDE7A protein

Recombinant human PDE7A was prepared as described previously [12]. The cDNA encoding human PDE7A (GeneBank accession no. Q13946) [28], amplified by polymerase chain reaction (PCR), was cloned in pFLAG expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden). The correct sequence of the resulting construct, pFLAG-PDE7A, was verified by sequencing. The plasmid was transfected into COS-7 cells by LipofectAMINE 2000 (Gibco BRL) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were washed with ice-cold phosphate-buffered saline and scraped in ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 2 mM magnesium acetate, 0.3 mM $CaCl_2$, 1 mM diethylenetriamine, 1.3 mM benzamide and 1 mM NaCl). The cell suspension was disrupted by a sonicator (TOMY Seiko, Tokyo, Japan) for 15 s (twice at 1-min intervals), and homogenates were centrifuged at $100000 \times g$ for 60 min. The resultant supernatant was collected and stored at $4^\circ C$ until use. The protein concentration of the supernatant was determined by a DC protein assay kit (Bio-Rad, CA, USA) using bovine serum albumin as a standard.

Assay of cAMP-PDE activity

PDE activity was determined by a modification of the method of Thompson et al. [29]. The assay buffer contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 0.33 mg/ml bovine

serum albumin, 22 mM $[^3H]$ cAMP and unlabelled cAMP. Reactions were started by adding 0.2–0.5 μ l enzyme solution to 500 μ l assay buffer, and incubated at $37^\circ C$ for 30 min. After boiling for 90 s, the mixture was added to 100 μ l of 1 mg/ml *Crotalus atrox* snake venom and incubated at $37^\circ C$ for 30 min. Reactions were stopped by the addition of 500 μ l methanol, and the resultant solutions were applied to Dowex ($1 \times 8-400$) columns. Aqueous scintillation mixture was added to each eluate, and radioactivity was measured with a scintillation counter. In evaluation of the effects of PDE inhibitors, the agents examined were dissolved in dimethyl sulphoxide. Assays were performed in triplicate at three or four different concentrations, the mean of the determinations at each concentration was plotted, and IC_{50} values were determined graphically.

Preparation of human PBMC

Adult male volunteers who were employees of Tanabe Seiyaku Co. were enlisted. All subjects gave written informed consent to the protocol as approved by the Company's ethics committee. As a result of preliminary examination, we decided to enroll nine donors whose PBMC produced IL-5 and proliferated in response to *Df* antigen in this study. No subjects were receiving medication. Heparinized venous blood was taken between 9 and 10 a.m. PBMC were prepared by Ficol-Paque density gradient centrifugation as described previously [20]. Cells were washed and suspended in AIM-V medium.

Cell cultures

PBMC suspended in AIM-V medium (2×10^6 /ml) were cultured in 24-well culture plates with or without *Df* for 6 days. In some experiments, PBMC were cultured with anti-CD3 MoAb (1 ng/ml) for the designated time periods. For cytokine assays, supernatants were harvested, and then frozen at $-70^\circ C$ until used. Each test compound was added at the start of culture.

Quantification of IL-5 in culture supernatants

Concentration of IL-5 in the culture supernatant was measured by enzyme-immunoassay (EIA). Purified rat antiserum/human IL-5 MoAb and biotinylated rat antihuman IL-5 MoAb were used as the capture and detection antibodies, respectively. The range of detection of the assay system was 2 pg/ml to 10 ng/ml.

Cell proliferation assay

After PBMC (2×10^6 /well) were cultured for 6 days with *Df* and test compound in 96-well flat-bottomed culture plates, proliferation was assessed by the bioreduction of tetrazolium salt into formazan as previously described [30] with Cell Titer 96™ Aqueous Non-Radioactive Cell Proliferation Assay kit according to the manufacturer's manual. Briefly, 20 μ l tetrazolium assay solution was added to 100 μ l cell culture in each well. After incubation for 4 h at $37^\circ C$, the absorbance of each well at 515 nm was measured. Results were expressed as stimulation index, which was calculated as the ratio of the absorbance in stimulated culture to that in control culture.

Cytokine messenger RNA (mRNA) expression

Gene expression of IL-2, IL-4 and IL-5 was analysed by the reverse transcription-polymerase chain reaction (RT-PCR) method, as reported previously [31]. Briefly, RNA was extracted from the cultured cells essentially following the one-step acid guanidinium isothiocyanate/phenol chloroform extraction method [32] using

ISOGEN. cDNA was synthesized from 1 µg cytoplasmic RNA using random primers and murine Moloney leukaemia virus reverse transcriptase. PCR was performed using the following RT-PCR amplimer sets.

IL-2	5'-CATGCACTAAGCTTTCGACTTGTCA-3'
	5'-CGTTGATATTCGTGATTAAGTCCTCG-3'
IL-4	5'-ATGGGTCACCTCCCAACTGCT-3'
	5'-CGAACACTTTGAATATTTCTCTCAT-3'
IL-5	5'-GCTTCGTCATTGAGTTTCTAGCT-3'
	5'-TGGCCGTCATATGATTTCTTTATTAAG-3'
β-actin	5'-ATGGATGATGATATCGCCGCG-3'
	5'-CTAAGACATTTGCGGTGGAC
	GATGGGGGCC-3'

To 50 µl (final volume) amplification solution (30 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl₂, 0.01% gelatin, 0.2 mM each deoxynucleotide triphosphate), 2 µl cDNA (corresponding to about 250 ng starting RNA material), 0.4 µM each primer, and 2 U GeneAmp® DNA polymerase were added. The mixture was heated at 95°C for 2 min, followed by 30 cycles, each consisting of incubation for 30 s at 95°C, 30 s at 60°C and 90 s at 73°C. The PCR products were analysed by 2% agarose gel electrophoresis in the presence of ethidium bromide. Expected sizes of PCR amplification products were 305, 456, 294, and 838 bp for IL-2, IL-4, IL-5 and β-actin, respectively.

Flow cytometric analysis of CD25 expression on cell surface of PBMC

After PBMC (2 × 10⁶/well) were cultured for 3 days with anti-CD3 MoAb (1 ng/ml), cells were harvested, washed and resuspended in staining buffer (PBS supplemented with 0.25% BSA and 0.1% NaN₃). After blocking with murine IgG for 1 h at 4°C, these cells were incubated with FITC-labelled anti-CD25 antibody or their control antibodies of appropriate isotype for 30 min at 4°C. In some experiments, cells were counter-stained with PE-labelled anti-CD3 antibody. After another two washes, cells were analysed using a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA, USA). Dead cells were gated out by their forward and angle light scatter profile. Data were analysed using the CellQuest® program.

Statistics

All data are presented as mean or mean ± s.e. Statistical analysis was performed by paired or Student's *t*-test for comparison between two groups and one-way ANOVA with Bonferroni's method for three groups or more. *P* < 0.05 was considered statistically significant.

RESULTS

Effects of PDE inhibitors on PDE7 activity

The first experiment was carried out to delineate the effects of T-2585 and RP 73401 on PDE7 activity, using purified recombinant human PDE7A. T-2585 inhibited PDE7A activity in a concentration-dependent manner (0.1–10 µM), and the IC₅₀ value of PDE7A inhibition by T-2585 was estimated to be 1.7 µM (Table 1). On the contrary, relatively weak suppression of PDE7A activity was obtained with the other PDE inhibitor, RP 73401, with maximum inhibition at 10 µM being only 35%.

Table 1. Effects of T-2585 and RP 73401 on PDE4 and 7 A activity

Isoenzyme	IC ₅₀ (µM)	
	T-2585	RP 73401
PDE4*	0.00013	0.00031
PDE7A	1.7	>10

Recombinant human PDE7A was purified from pFLAG-PDE7A-transfected COS-7 cells and incubated in the presence of [³H]-cAMP with or without various concentrations of T-2585 or RP 73401. The radioactivity of hydrolysed [³H]-cAMP was then measured. IC₅₀ values were determined graphically from a concentration-inhibition curve. *Data are cited from our previous report [25].

Effects of PDE inhibitors on IL-5 production by PBMC

The effects of T-2585 and RP 73401 on Df-induced IL-5 production by PBMC were determined. PBMC were incubated with Df, and the resulting supernatants were assayed for IL-5. IL-5 was produced spontaneously by PBMC of several individuals, while there was a significant increase in IL-5 production upon stimulation with Df, and the antigen-specific IL-5 production reached a maximum on day 6 (Fig. 1a). The optimal concentration of antigen was 1–10 µg/ml in most subjects. Other cytokines, IL-2 and IL-4, were hardly detected in the culture supernatants of PBMC incubated with or without antigen. T-2585 suppressed antigen-induced IL-5 production in a concentration-dependent manner (0.1–10 µM) (Fig. 1b). However, RP 73401 caused little inhibition of IL-5 production by PBMC even at 10 µM (22%). No concentrations of T-2585 or RP-73401 used in this study displayed cytotoxicity to PBMC (data not shown). We have previously demonstrated that IL-5 produced by PBMC is derived exclusively from CD4⁺ T cells [31], suggesting that T-2585 suppresses IL-5 production by CD4⁺ T cells.

Effects of PDE inhibitors on proliferation of PBMC

The effects of T-2585 and RP 73401 on another antigen-specific response of PBMC, proliferation, were examined next. As an antigen-specific proliferative response of PBMC was detected on day 2 and reached a maximum on day 6, proliferation was assessed on day 6. The stimulation index was highest at an antigen concentration of 10 µg/ml (2.1 ± 0.22, *n* = 6). As shown in Fig. 2, antigen-specific proliferation of PBMC was inhibited by T-2585 in a concentration-dependent manner. On the other hand, the maximum inhibition of PBMC proliferation obtained with 0.1–10 µM RP 73401 was only 20%. Almost 90% of the living cells present after 6 days of culture with Df antigen were determined to be CD4⁺ T cells by flow cytometric analysis (data not shown), suggesting that T-2585 inhibited the proliferation of CD4⁺ T cells.

Effects of PDE inhibitors on cytokine mRNA expression in PBMC

The dose of T-2585 most effective at inhibiting proliferation (Fig. 2) was similar to that capable of inhibiting IL-5 production (Fig. 1), suggesting that the attenuation of IL-5 production by T-2585 was caused at least in part by the reduction in PBMC numbers. In order to evaluate whether T-2585 has a direct effect on cytokine synthesis, expression of cytokine mRNA in PBMC upon

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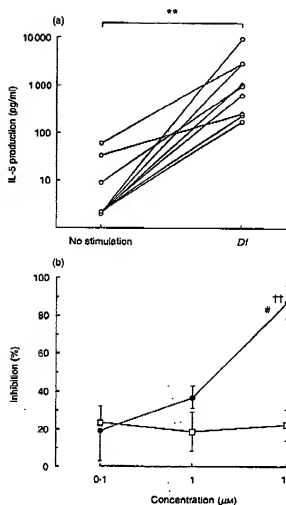


Fig. 1. Effects of T-2585 and RP 73401 on IL-5 production by human PBMC. PBMC (2×10^6 /ml) were incubated with or without Df ($10 \mu\text{g/ml}$) for 6 days. Various concentrations of T-2585 (●) or RP 73401 (□) were included throughout the culture period. The quantity of IL-5 in the culture supernatants was measured by EIA. Data from each individual subject (a) and the percentage inhibition of Df-induced IL-5 production by PBMC (b) are shown ($n = 4-9$). ** $P < 0.01$ (paired *t*-test), # $P < 0.05$, compared with control cultures (Bonferroni's method), †† $P < 0.01$, compared with RP 73401 (Student's *t*-test).

stimulation with anti-CD3 MoAb was examined. The CD3 molecule is associated with the T cell receptor, and the stimulation signal induced by anti-CD3 MoAb passes through this receptor. Anti-CD3 MoAb was therefore used to mimic the stimulus produced by a specific antigen. No obvious expression of any cytokine mRNA was obtained in unstimulated PBMC. Expression of IL-5 mRNA was induced in PBMC upon anti-CD3 MoAb (1 ng/ml) stimulation, and expression reached a maximum at 6 h (Fig. 3). Interestingly, mRNA for two other T cell cytokines, IL-2 and IL-4, was also expressed clearly upon anti-CD3 MoAb stimulation, even though neither cytokine was detected in the culture supernatant of PBMC stimulated with Df. IL-2 and IL-4 are T cell

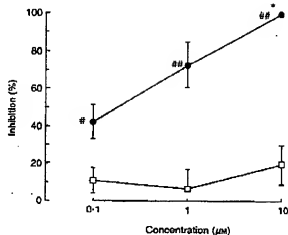


Fig. 2. Effects of T-2585 and RP 73401 on the proliferation of human PBMC. PBMC (2×10^6 /ml) were incubated with Df ($10 \mu\text{g/ml}$) in the presence or absence of T-2585 (●) or RP 73401 (□) for 6 days. The proliferation of PBMC was measured by a non-radioactive cell proliferation assay system. The percentage inhibition of Df-induced proliferation of PBMC is shown ($n = 4$). * $P < 0.05$, compared with control cultures (Bonferroni's method), # $P < 0.05$, compared with RP 73401 (Student's *t*-test).

and/or B cell growth factors [33,34], and receptors for these two cytokines are expressed abundantly on the cell surface of PBMC, suggesting that both cytokines produced by peripheral T cells are quickly trapped by these receptors.

As shown in Fig. 3, $10 \mu\text{M}$ T-2585 significantly diminished IL-2, IL-4 and IL-5 mRNA expression in anti-CD3 MoAb-stimulated PBMC. Suppression of mRNA expression of these cytokines by T-2585 was obtained even at $1 \mu\text{M}$ (data not shown). To confirm the specificity of the effect of T-2585, we next examined the possibility that T-2585 affects cytokine mRNA expression initiated by phorbol-ester and calcium ionophore. mRNA expression of IL-2, IL-4 and IL-5 was significantly up-regulated by stimulation with phorbol 12-myristate 13 acetate (PMA) + ionomycin. T-2585 ($10 \mu\text{M}$) did not, however, affect this PMA + ionomycin-induced cytokine mRNA expression at all (Fig. 3). Neither anti-CD3 MoAb nor PMA + ionomycin-stimulated IL-2, IL-4 and IL-5 mRNA expression was affected by $10 \mu\text{M}$ RP 73401.

These findings suggest that T-2585 inhibits T cell receptor-mediated production of not only IL-5, but also IL-2 and IL-4 in human peripheral T cells at a point in the upstream signalling pathway leading to the regulation of gene transcription.

Effects of PDE inhibitors on CD25 expression of PBMC

We also examined the effects of T-2585 and RP 73401 on another T cell activation marker, CD25. CD25 is the IL-2 receptor α -chain and is expressed on the T cell surface membrane following cell activation. After PBMC had been stimulated with anti-CD3 MoAb, CD25 expression was determined by flow cytometry. CD25 was hardly expressed on the cell surface of unstimulated PBMC but its expression was clearly up-regulated by anti-CD3 MoAb (1 ng/ml) stimulation, reaching a maximum after 3 days of stimulation ($21 \pm 8.1\%$ of PBMC, $n = 3$; Fig. 4a). As 60–70% of the CD25⁺ cells in PBMC were determined to be CD3⁺, (data not shown), the effect of the PDE inhibitors on CD25 expression was

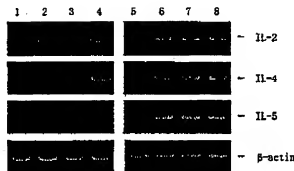


Fig. 3. Effects of T-2585 and RP 73401 on cytokine mRNA expression in human PBMC. PBMC ($4 \times 10^6/\text{ml}$) were incubated with anti-CD3 antibody (1 ng/ml) or PMA (20 nM) + ionomycin (1 μM) with or without T-2585 or RP 73401 for 6 h. Total RNA was then extracted, reverse transcribed and amplified by PCR. Lanes 1 and 5: no stimulation; 2-4: anti-CD3 antibody stimulation; 6-8: PMA + ionomycin stimulation; 3 and 7: $10 \mu\text{M}$ T-2585; 4 and 8: $10 \mu\text{M}$ RP 73401. This is one representative result from a total of three separate experiments.

determined after the T cells in PBMC were gated by counter-staining with a PE-labelled CD3 antibody. As shown in Fig. 4b, T-2585 suppressed anti-CD3 MoAb-induced CD25 expression on human T cells in a concentration-dependent manner (0.1–10 μM). On the other hand, relatively weak inhibition of CD25 expression was obtained with RP 73401 (24–33 %).

DISCUSSION

Our findings demonstrate that the PDE inhibitor, T-2585, acts on human peripheral T cells to suppress: IL-5 protein production; IL-2, IL-4 and IL-5 mRNA expression; cell proliferation; and CD25 expression in the dose range at which the drug inhibits PDE7A activity. However, another potent PDE inhibitor RP 73401, which does not inhibit PDE7A activity, effectively ($\text{IC}_{50} > 10 \mu\text{M}$), suppressed only these T cell responses weakly at $10 \mu\text{M}$. Since the IC_{50} of RP 73401 for PDE4 suppression is 0.00031 μM , this suggests that PDE7, but not PDE4, plays a substantial role in regulating human T cell function.

cAMP-elevating agents affect T cell function [1,4,35,36] and the inhibition of human cytokine production by such agents has been documented [5,6,20]. Prostaglandin E_2 , forskolin and a cAMP-analogue, dibutyryl cAMP, have been shown to suppress IL-2, IL-4 and IL-5 production by T cell receptor-stimulated PBMC from asthmatic asthmatics [20]. The suppression of both CD25 expression and proliferation of human T cells by prostaglandin E_2 and other cAMP-affecting agents has also been reported [7,35,37,38]. In addition, several selective and nonselective PDE inhibitors suppress human T cell responses such as proliferation, mRNA expression and protein synthesis of cytokines [34,16–23]. We have reported previously that a PDE inhibitor that is structurally related to T-2585 inhibited cAMP-PDE activity in human PBMC and subsequently increased the level of intracellular cAMP [20]. Several groups have reported that cAMP suppresses Ca^{2+} release from intracellular stores [39,40]. Such suppression is mediated by the cAMP-activated protein kinase PKA acting on the signalling pathway downstream of the T cell

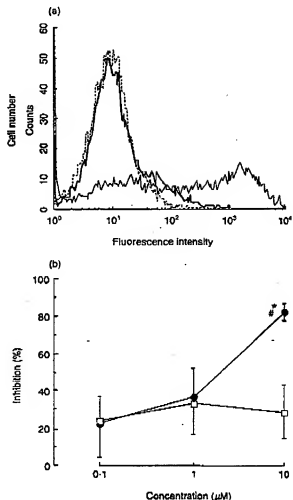


Fig. 4. Effects of T-2585 and RP 73401 on CD25 expression of human T cells. PBMC ($2 \times 10^6/\text{ml}$) were incubated with anti-CD3 MoAb (1 ng/ml) with or without T-2585 (●) or RP 73401 (□) for 3 days. CD25 expression on PBMC was analysed by flow cytometry. (a) The histogram of PBMC correspond to representative experiments with (solid line) or without (bold line) stimulation. The dotted line indicates baseline fluorescence obtained with FITC-labelled control antibody. (b) Percentage inhibition of anti-CD3 MoAb-induced CD25 expression on CD3⁺ cells is shown ($n = 3$). * $P < 0.05$, compared with control cultures (Bonferroni's method). * $P < 0.05$, compared with RP 73401 (paired t -test).

receptor [40,41]. We demonstrated recently that prostaglandin E_2 suppresses both intracellular Ca^{2+} elevation and IL-5 production of human T cells stimulated by anti-CD3 MoAb [42]. This suggests that the suppression of T cell function by cAMP is caused, at least in part, by down-modulation of intracellular Ca^{2+} influx. This is consistent with the result that cytokine mRNA expression in PBMC induced by anti-CD3 MoAb, but not PMA + ionomycin, is suppressed by T-2585. Taken together, it is reasonable to propose that the inhibition of PDE7 activity by T-2585 results in the suppression of T cell function as a consequence of the increase in the level of intracellular cAMP.

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Recently, Li *et al.* reported that PDE7 is induced in T cells upon stimulation with anti-CD3 and anti-CD28 antibody [43]. The significance of PDE7 for T cell function was implicated by the fact that a selective reduction in PDE7 expression by antisense oligonucleotide inhibits T cell proliferation. Our present findings support these findings and further demonstrate that PDE7 may modulate those T cell functions induced by stimulation with a specific antigen.

In apparent contradiction to our present findings, a number of earlier studies have demonstrated that PDE4 plays a role in the regulation of multiple T cell functions by using the selective PDE4 inhibitor, rolipram, which does not inhibit PDE7 activity [13,14,16-19]. However, in some reports, the effective dose of rolipram required to inhibit T cell activation was higher than that required to suppress PDE4 activity [21-23]. Interestingly, in our experiments, RP 73401 displayed very little suppression (10-30%) of most of the T cell functions examined (Figs 1-4) in the dose range at which the drug inhibits the activity of PDE4, but not PDE7A, completely (0.1-1 μ M). Therefore, PDE4 may have a supplemental role in the regulation of the intracellular cAMP level in human T cells, and consequently in T cell function. It is possible that T-2585 inhibits the variety of T cell receptor initiated T cell functions via the combined effects on PDE4 and PDE7.

From our findings, a possible contribution of PDE3 inhibition to T-2585-mediated suppression of T cell function has not been completely ruled out, since the inhibitory potency of T-2585 on PDE3 activity isolated from guinea pig heart was similar to that on PDE7A activity [25]. Nevertheless, previous reports from at least three separate groups demonstrating that PDE3 inhibitors failed to affect cytokine mRNA expression and proliferation of human PBMC [16-19,22,23] rule against a critical role for PDE3 in T cell function. We also confirmed by preliminary experiments that IL-5 production was not inhibited by CI-930, a PDE3 inhibitor, in our experimental system (data not shown). In order to elucidate additional details of the comparative roles of PDE7 and other PDE isoenzymes in T cell function, it will be necessary to delineate the effects of currently existing PDE inhibitors on PDE7 activity in human T cells. Most importantly, the generation of a selective PDE7 inhibitor will be eagerly awaited.

It is significant that T-2585 but not RP 73401 effectively suppressed IL-5 production by PBMC because accumulating evidence suggests that IL-5 is the key cytokine involved in allergic diseases, such as asthma and atopic dermatitis, associated with eosinophilic inflammation. IL-5 is a lymphokine produced primarily by activated T cells and it enhances the proliferation, differentiation and survival of eosinophils [44,45]. Activated T cells expressing IL-5 mRNA were found in increased numbers in the bronchial mucosa of asthmatic patients [46] and the numbers were further increased upon antigen challenge [47]. Administration of an anti-IL-5 neutralizing antibody abrogated allergic eosinophilic inflammation in experimental allergic models [48-50]. Our present findings suggest that selective inhibitors for PDE7, but not PDE4, are promising drugs for the management of chronic allergic disorders. Furthermore, PDE7 inhibitors able to down-regulate total T cell function have the potential for treating a wide range of diseases associated with an abnormal T cell response.

In conclusion, PDE7 has the potential to regulate human T cell functions including cytokine production, proliferation and expression of activation markers. This suggests the possible man-

agement of various immunological diseases by treatment with selective PDE7 inhibitors.

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Phosphodiesterase profile of human B lymphocytes from normal and atopic donors and the effects of PDE inhibition on B cell proliferation

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1 CD19⁺ B lymphocytes were purified from the peripheral blood of normal and atopic subjects to analyse and compare the phosphodiesterase (PDE) activity profile, PDE mRNA expression and the importance of PDE activity for the regulation of B cell function.

2 The majority of cyclic AMP hydrolyzing activity of human B cells was cytosolic PDE4, followed by cytosolic PDE3-like activity; marginal PDE3 activity was found only in the particulate B cell fraction. PDE1, PDE2 and PDE5 activities were not detected.

3 By cDNA-PCR analysis mRNA of the PDE4 subtypes A, B (splice variant PDE4B2) and D were detected. In addition, a weak signal for PDE3A was found.

4 No differences in PDE activities or mRNA expression of PDE subtypes were found in B cells from either normal or atopic subjects.

5 Stimulation of B lymphocytes with the polyelonal stimulus lipopolysaccharide (LPS) induced a proliferative response in a time- and concentration-dependent manner, which was increased in the presence of Interleukin-4 (IL-4). PDE4 inhibitors (rolipram, plicamylast) led to an increase in the cellular cyclic AMP concentration and to an augmentation of proliferation, whereas a PDE3 inhibitor (metapirone) was ineffective, which is in accordance with the PDE profile found. The proliferation enhancing effect of the PDE4 inhibitors was partly mimicked by the cyclic AMP analogues dibutyryl (db) cyclic AMP and 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole-3',5'-cyclic monophosphorothioate, Sp-isomer (dcl-cBIMPS), respectively. However, at concentrations exceeding 100 μ M db-cyclic AMP suppressed B lymphocyte proliferation, probably as a result of cytotoxicity. Prostaglandin E₂ (PGE₂, 1 μ M) and forskolin (10 μ M) did not affect B cell proliferation, even when given in combination with rolipram.

6 Inhibition of protein kinase A (PKA) by differentially acting selective inhibitors (KT 5720, Rp-8-Br-cyclic AMPs) decreased the proliferative response of control cells and reversed the proliferation enhancing effects of rolipram.

7 Importantly, PDE4 activity in LPS/IL-4-activated B lymphocytes decreased by about 50% compared to unstimulated control values.

8 We conclude that an increase in cyclic AMP, mediated by down-regulation of PDE4 activity, is involved in the stimulation of B cell proliferation in response to LPS/IL-4. B cell proliferation in response to a mitogenic stimulus can be further enhanced by pharmacological elevation of cyclic AMP.

Keywords: CD19⁺ B cells; atopic dermatitis; PDE4 subtypes; rolipram; cyclic AMP; PDE isoenzymes; protein kinase A; PKA inhibitors

Introduction

Among the large variety of cellular responses that are orchestrated by the adenosine 3',5'-cyclic monophosphate (cyclic AMP)/protein kinase A (PKA) pathway, the anti-inflammatory properties of this second messenger system have attracted particular interest during recent years. Cyclic AMP is synthesized through the action of adenylyl cyclase and its sole route of degradation is by the action of cyclic AMP phosphodiesterases (PDE), a multigene family (for recent reviews see Beavo *et al.*, 1994; Bolger, 1994; Beavo, 1995; Loughney & Ferguson, 1996). In inflammatory cells, members of the low K_m cyclic AMP-specific PDE4 are the predominant isoenzymes in terms of expression and distribution. PDE4 is believed to play an important immunoregulatory role, since many inflammatory reactions are suppressed by PDE4-selective inhibitors such as rolipram, which is in accordance

with the long-known anti-inflammatory properties of cyclic AMP elevating agents and cyclic AMP itself. In monocytes, the production of proinflammatory mediators is drastically decreased (Semmler *et al.*, 1993; Prabhakar *et al.*, 1994; Seldin *et al.*, 1995). Similarly, various functions of other leucocytes including eosinophils (Dent *et al.*, 1994; Hatzelmann *et al.*, 1995; Soumess *et al.*, 1995; Tenor *et al.*, 1996), neutrophils (Wright *et al.*, 1990; Schudt *et al.*, 1991; Fontsch *et al.*, 1993), macrophages (Schade & Schudt, 1993; Fischer *et al.*, 1993; Gantner *et al.*, 1997a), basophils (Peachell *et al.*, 1992), mast cells (Torphy *et al.*, 1992) and T lymphocytes (Roblesek *et al.*, 1991; Essayan *et al.*, 1994; Crocker *et al.*, 1996; Gantner *et al.*, 1995; Gienbycz *et al.*, 1996) are strongly impaired. B cells as well as eosinophils even undergo apoptosis following cyclic AMP elevation/PDE inhibition (Lemo *et al.*, 1995; Hallsworth *et al.*, 1996).

In view of these anti-inflammatory properties of cyclic AMP, it is not surprising that there is considerable interest in

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the development of PDE4 inhibitors for the treatment of chronic inflammatory diseases such as asthma and atopic dermatitis, where all of the inflammatory cell populations mentioned above play a critical role. Moreover, it has been found that in leukocytes of atopic patients a higher PDE4 activity exists (Grewe *et al.*, 1982; Chan *et al.*, 1993) and that peripheral blood mononuclear cells (PBMC) from such patients are more sensitive to PDE4 inhibitors (Chan & Haniffa, 1993; Banner *et al.*, 1995). This provides a further rationale for the development of PDE4 inhibitors as anti-inflammatory drugs.

However, many studies on PDE isoenzyme distribution and functional consequences of PDE inhibition in leukocytes have been performed in mixed cell populations such as PBMC. This prompted us to analyse individually, and to characterize functionally highly purified populations of human freshly isolated inflammatory cells relevant in asthmatic and atopic disorders. Analysis of monocytes and macrophages, neutrophils, eosinophils and CD4⁺ as well as CD8⁺ T lymphocytes confirmed the prominent role of PDE4 isoenzymes for the regulation of inflammatory responses in these cells (for review see Tenor & Schudt, 1996; Dent & Glembycz, 1996).

In B cells, both stimulating and inhibitory effects have been attributed to cyclic AMP elevation (Holt *et al.*, 1988; Roper & Phipps, 1992; Garrone & Banchereau, 1993; Coqueret *et al.*, 1996). Whereas β -adrenergic agonists, adenylyl cyclase activators, prostaglandin E₂ (PGE₂) and cyclic AMP analogues have been studied with regard to B cell proliferation and function, the effects of PDE isoenzyme-selective inhibitors have not yet been determined. For human CD19⁺ B lymphocytes, details of the PDE isoenzyme profile and the functional consequences of PDE inhibition are not known. Thus, in the present study we used highly purified B lymphocytes from both healthy donors and atopic patients and compared their PDE mRNA expression and activity profile under control and stimulated conditions. Furthermore, the influence of selective PDE inhibitors on polyclonal B cell proliferation was studied.

Methods

Subjects

Normal healthy donors who had no history of asthma, allergic rhinitis or atopic dermatitis were selected for this study. Additionally, the blood of a separate group of patients suffering from atopic dermatitis was used. The patients were off medication (corticosteroids) for at least 3 weeks before the investigation. This study was approved by the local ethics committee and carried out according to national guidelines.

Cell preparation

For each experiment 250 ml of peripheral venous blood was drawn from a single volunteer. Citrate (0.31%) was used as an anti-coagulant. The blood was diluted 1:6 fold with PBS (pH 7.4) before centrifugation at 220 \times g at 20°C for 20 min. The cell pellet was layered on a Percoll gradient (p 1.077 g ml⁻¹) and the interphase containing the peripheral blood mononuclear cells (PBMC) was obtained following centrifugation at 800 \times g. Cells were washed twice in elutriation medium (PBS, 2% heat-inactivated human AB serum, 2 mM EDTA, 5 mM glucose, pH 7.4) before countercentrifugation elutriation of the cells with a J2-MC centrifuge equipped with a JE-68 rotor (Beckman, U.S.A.). The lymphocyte containing fraction

(platelet-free, >95% purity) was obtained at a flow rate of 29 ml min⁻¹ and a rotor speed of 3000 r.p.m. Pure B lymphocytes were obtained by positive selection using magnetic-beads-conjugated anti-CD19 Ab and detaching Ab according to the manufacturer's protocol (Dyna, Oslo, Norway). B cell purity (CD19⁺ cells) was checked by flow cytometry (Coulter, Hamburg, Germany) and was always >99%. No CD3⁺ T cells or CD14⁺ monocytes were detectable which is in accordance to the unresponsiveness of these cells towards the T cell mitogen, phytohemagglutinin (PHA, data not shown).

Preparation of subcellular B lymphocyte fractions

Freshly prepared CD19⁺ B cells were resuspended at 10⁷ cells ml⁻¹ in homogenization buffer (10 mM HEPES, pH 8.2, 1 mM β -mercaptoethanol, 1 mM MgCl₂, 1 mM EGTA, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 5 μ M pepstatin A, 10 μ M leupeptin, 50 μ M PMSF, 10 μ M soybean trypsin inhibitor and 2 mM benzamide) and disrupted by sonication (Branson 250 sonifier, output control 10%, duty cycle 1). The complete disruption of the cells (>98%) was checked via trypan blue exclusion or, alternatively, via lactate dehydrogenase (LDH) release. The homogenate was spun at 1000 \times g for 5 min to remove viable cells (1–2%) and nuclei. The samples were then centrifuged at 100,000 \times g for 60 min at 4°C. The supernatant was decanted and the pellet was resuspended in the corresponding volume of homogenization buffer. These two fractions (further referred to as the soluble and particulate fraction, respectively) were stored at –80°C until further use.

PDE activity assay

PDE activity was determined as described by Thompson *et al.* (1979) with some modifications (Bauer & Schwabe, 1980). In brief, the enzyme containing fractions were assayed in a final volume of 200 μ l containing 60 mM Tris HCl, pH 7.4, 5 mM MgCl₂, 0.5 μ M cyclic AMP or cyclic GMP (28 000 c.p.m. [³H]-cyclic AMP or [³H]-cyclic GMP) and were incubated in the presence or absence of activators or inhibitors for 30 min at 37°C. The reaction was terminated by the addition of 50 μ l 0.2 N HCl and the assay mixture was left on ice for a further 15 min. *Crotalus atrox* snake venom (0.5 mg ml⁻¹) was added for 15 min at 37°C and the assay mixture was then loaded onto QAE-Sephadex A-25 columns (1 ml bed volume) and eluted with 2 ml ammonium formate (30 mM, pH 6.0). The radioactivity in the eluate was counted in a liquid scintillation counter (Beckman). PDE isoenzyme activity calculations in the soluble as well as in the particulate B cell fractions were performed, making use of activators and the PDE isoenzyme-selective inhibitors metoprolol (PDE3), rolipram (PDE4) and zaprinast (PDE5), as described by Tenor *et al.* (1995a). PDE7 activity was defined as the residual cyclic AMP hydrolyzing activity in the presence of metoprolol (1 μ M) and rolipram (10 μ M), respectively.

PDE expression analysis by cDNA-PCR

Preparation of RNA, conditions for cDNA-PCR with the thermocycler 60/2 from Bio-med (Therex, Germany), and separation of PCR products by polyacrylamide gel electrophoresis were performed as described in previous studies (Willech *et al.*, 1993; Beck *et al.*, 1995). The buffer for PCR provided by the supplier (Amersham) was applied as recommended. After the staining of the gels with ethidium

bromide, the signals were digitized by the CS-1 videomager (Cybertech, Berlin, Germany). Signal intensities calculated with the WINCAM densitometric software (Cybertech) assigned to the various genes of interest were referred to those corresponding to the β 2-microglobulin (β 2m) gene obtained by a simultaneously performed PCR with an aliquot of the same cDNA.

The following primers for detecting PDE mRNA expression were used: PDE3A (sense: CAATCTCTAT-GATT-CAGCA, position 3061 to 3079; antisense: CTGG-TCTGGGCTTTGGGTT, position 3427 to 3443; genebank accession# M91667); PDE3B (sense: TCTATATCTT-TCTCGCCAG, position 2580 to 2598; antisense: CTCTTCATCTCCCTGCTC, position 2881 to 2899; genebank accession# U38178); PDE4A (sense: TCAGAGCTGGC-GCTTATGTAC (adopted from Engels *et al.*, 1994, primer PE21), position 1582 to 1602; antisense: CCGTATGCTTGT-CACA CAT (adopted from Engels *et al.*, 1994, primer PE32), position 2002 to 2020; genebank accession# L20965); PDE4B2 (sense: TGCTATGGACAGCCTGAC, position 341 to 359; antisense: TGTGAGAATATCCAGCC (adopted from Obernolte *et al.*, 1993), position 830 to 846, genebank accession# M97515); PDE4C (sense: ATGAGGAGGAAGAAGAGGA-GGGG; position 2117 to 2139; antisense: AGTCTCT-CGCTGTGTCAGG; position 2229 to 2247, genebank accession# Z46632); PDE4D (sense: GCAAGATCGAGCA-CCTAGCA, position 461 to 480; antisense: ACCAGACAA-CTCTGCTATTC, position 932 to 971, genebank accession# L20970, according to Engels *et al.*, 1994); PDE7 (sense: CTC-TAGTAAGCTTAA, position 557 to 571; antisense: GGCTT-ATCTACATCTG, position 939 to 956, genebank accession# L12052, according to Michael *et al.*, 1993).

With the exception of the PDE4B primer, which detects the short form PDE4B2 variant only, the PDE primers we used were generic, i.e. they amplify all splice variants of a certain gene. The primers for the β 2m gene were adopted from a previous study (Wilsch *et al.*, 1993). To determine the appropriate number of cycles for a semiquantitative estimation of the PCR products, the exponential range (number of PCR cycles versus signal intensity) of polymerase chain reaction under the chosen conditions was determined for each pair of primers. Accordingly, for the semiquantitative cDNA-PCR analysis the following number of cycles were used: 26 cycles (PDE3A), 28 cycles (PDE3B), 30 cycles (PDE4A), 28 cycles (PDE4B2), 28 cycles (PDE4C), 28 cycles (PDE4D), 28 cycles (PDE7), 19 cycles (β 2m). The amplifiers used for analysis of PDE4A expression also detect the PDE4C gene, which can be differentiated with restriction enzyme digest analysis according to Engels *et al.* (1994).

Cyclic AMP measurements

Intracellular cyclic AMP content was determined by an enzyme immunoassay (Biotrak EIA) according to the non-acetylation assay instructions provided by the supplier (Amersham Life Sciences, Braunschweig, Germany) with the modification of adding the unselective PDE inhibitor isobutyl methyl xanthine (IBMX; 0.5 mM) to the extraction buffer to ensure inhibition of cyclic nucleotide breakdown by PDE activity.

B cell proliferation

B cells (2×10^6 per well containing 200 μ l RPMI 1640 medium) were seeded in 96-well flat bottom plates in the presence of test compounds or the corresponding volume of vehicle (dimethylsulphoxide; DMSO, 0.1% final concentration). Thirty minutes

later, cells were stimulated by the indicated concentrations of lipopolysaccharide (LPS) and interleukin-4 (IL-4) for 5 days. Proliferation was evaluated by standard liquid scintillation counting based on [3 H]-thymidine uptake which was present for the last 24 h of culture (0.2 μ Ci/well).

Cell culture media and reagents

RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 μ g/ml) was used for all cultures. Purified rhIL-4 was purchased from ICC Chemicals (Ismaning, Germany). Moutapione was kindly provided by Neutmann (Köln, Germany), and rolipram was obtained from Schering (Berlin, Germany). Rp-8-Br-cyclic AMPs and 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole-3',5'-cyclic monophosphorothioate (dcl-cBIMPS) were from Biolog (Bremen, Germany), and KT 5720 was from Biomol (Hamburg, Germany). Lipopolysaccharide (LPS; *S. abortus equi*) and all reagents not further specified were purchased from Sigma (Deisenhofen, Germany).

Statistics

Unless otherwise stated, data are expressed as means \pm s.d. of results carried out in at least three independent experiments with blood from different donors. Statistical significances were determined with unpaired Student's *t* test if applicable or according to Welch's approximate *t* solution if variances were nonhomogeneous by use of commercially available statistical programmes (GraphPad Software, San Diego, CA). *P* < 0.05 was considered as statistically significant.

Results

PDE activity of resting human CD19⁺ B lymphocytes from normal and atopic donors

An increased cyclic AMP hydrolyzing activity in leucocytes of atopic patients has been found previously (reviewed in Hanifin & Chan, 1995). However, in pure human B cells the PDE profile has not been analysed previously. This prompted us to determine PDE activity and PDE mRNA expression of B lymphocytes prepared from peripheral blood of normal and atopic individuals. Most of the cyclic AMP hydrolyzing activity of the soluble (cytosolic) fraction was inhibited by rolipram (10 μ M) and therefore represents PDE4 activity. In addition, substantial PDE7 activity (residual activity in the presence of a PDE4 and a PDE3 inhibitor) was found, which was more prominent in the soluble compared to the particulate fraction. Overall, only marginal activity of PDE3 (moutapione-sensitive) was found, which was predominantly membrane-bound. Neither PDE1, PDE2 nor PDE5 activity were detectable in purified human B cells. The PDE activity profile was identical between normal (Figure 1a) and atopic individuals (Figure 1b).

To characterize PDE isoenzyme and subtype mRNA expression, we prepared mRNA from both normal and atopic B cells for cDNA-PCR analysis. As expected from the PDE activity profile, mRNA transcripts for PDE3, PDE4 and PDE7 were found (Figure 2). Among the known PDE3 subtypes, a weak PDE3A mRNA signal was noted, but no PDE3B mRNA was detectable. PDE4A, 4B2 and 4D mRNAs were present, but PDE4C was lacking in B cells. A very high mRNA signal was obtained for PDE7.

Again, no significant differences in PDE mRNA expression between B cells from healthy and atopic donors were noted (Figure 2). Since the PDE profile of B cells from atopic and healthy donors turned out to be identical, the further studies were carried out with cells from healthy volunteers.

Increased DNA synthesis following PDE4 inhibition in B cells stimulated by a combination of LPS and IL-4

To investigate functional consequences of PDE inhibition in human B cells, we tested the influence of PDE inhibitors on B cell proliferation. Based on the PDE isoenzyme pattern described previously, we concentrated on PDE4 and PDE3 selective compounds. Due to the lack of PDE7 selective inhibitors, the question of a possible regulatory role of this PDE family was not addressed.

In the first set of experiments, B cell proliferation was induced over various time periods (between 2 and 9 days) by polyclonal stimulation with combinations of LPS (0.1–10 $\mu\text{g ml}^{-1}$) and IL-4 (10–1000 u ml^{-1}). At day 5, stimulation with LPS (10 $\mu\text{g ml}^{-1}$) plus IL-4 (100 u ml^{-1}) was optimal with regard to [^3H]-thymidine incorporation (Figure 3), and therefore was used for the following experiments.

The prototype PDE4-selective inhibitor rolipram concentration-dependently increased the proliferative response of IL-4/LPS-stimulated CD19⁺ B cells. A two fold higher thymidine incorporation rate was observed at concentrations $\geq 1 \mu\text{M}$ rolipram (Figure 4). Analogous results were obtained with piclamilan, another PDE4-selective compound (210 \pm 23% proliferation at 1 μM , $n=4$). In contrast, the PDE3 inhibitor metoprolol neither modulated B cell proliferation nor

influenced the effect of the PDE4 inhibitor (Figure 4). Thus, PDE3 inhibition does not play a significant functional role in B cell proliferation, which is in agreement with the marginal PDE3 expression and activity present in these cells (see Figures 1 and 2).

Since PDE inhibitors are assumed to act via elevation of cyclic AMP, we measured the intracellular concentration of this second messenger. Indeed, a three fold increase in the cyclic

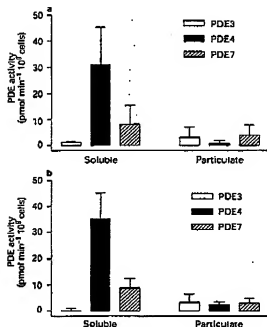


Figure 1 PDE activity profile of resting human B lymphocytes. PDE activities were determined in the soluble and particulate fraction of highly purified B cells isolated from normal (a) or atopic donors (b). Values were calculated on a per cell basis. Data representing PDE3, PDE4 and PDE7 are expressed as mean \pm s.d. from 5 independent preparations. No PDE1, PDE2 or PDE5 was detected.

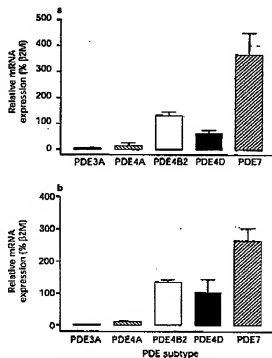


Figure 2 Relative PDE3, PDE4 and PDE7 subtype mRNA expression analysis by cDNA-PCR of resting human B cells from (a) normal or (b) atopic donors. Data are expressed as mean values \pm s.d. from 5–6 independent preparations.

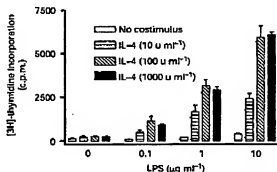


Figure 3 Polyclonal human B cell proliferation in response to LPS plus IL-4. B cells (2×10^5) were incubated in 200 μl culture medium with LPS in the absence or presence of various concentrations of IL-4. Four days later, [^3H]-thymidine was added (0.2 μCi per well) for another 24 h. Cells were then harvested and proliferation was determined by [^3H]-thymidine incorporation. Data represent mean values \pm s.d. of 4 independent experiments.

AMP levels was found 1 h after stimulation of the cells in the presence of rolipram compared to unstimulated controls or LPS/IL-4-treated cells (Table 1). We do not know whether, and, if so, to what extent and at what time point any significant changes in the cyclic AMP concentration were induced following LPS/IL-4 stimulation. Due to cell number limitations, the low cyclic AMP content of B cells, and the long experimental procedure we did not further expand these experiments.

We also examined the effect of the cell-permeable cyclic AMP analogues dibutyryl cyclic AMP (db-cyclic AMP) and dd-cBIMPS on B cell proliferation. A bell-shaped concentration-response curve was obtained for db-cyclic AMP. At concentrations up to 100 μ M both compounds mimicked the effect of the PDE4 inhibitors and increased IL-4/LPS-induced DNA synthesis rate by about 30% compared to control cells (Figure 4). However, at concentrations >300 μ M, db-cyclic AMP strongly suppressed the proliferation of CD19⁺ B cells (50–60% reduction, not shown) indicating that at least two cyclic AMP-dependent pathways might be involved in the regulation of DNA synthesis of human B cells. Trypan blue exclusion studies revealed evidence for the induction of cytotoxicity in the presence of 1 mM cyclic AMP after 5 days of LPS/IL-4 stimulation (68 \pm 7% viable cells in the presence, 83 \pm 5% viable cells in the absence of db-cyclic AMP; $n=5$,

$P<0.05$), providing an explanation for the proliferation suppressing effects of high db-cyclic AMP concentrations.

In contrast to db-cyclic AMP and dd-cBIMPS, both forskolin (0.01–10 μ M), an adenylate cyclase activator, and PGE₂ (0.001–1 μ M) lacked efficacy (Figure 4). Moreover, both compounds failed to alter the concentration-response curve of rolipram at 10 μ M (forskolin) or 1 μ M (PGE₂), respectively (data not shown).

PKA activity is necessary for B cell proliferation

The influence of PKA inhibitors on LPS/IL-4-induced B lymphocyte proliferation in the absence or presence of rolipram was investigated. Remarkably, a dramatic inhibition of the proliferative response was observed under both experimental conditions. The two differentially acting PKA inhibitors tested (KT 5720, Rp-8-Br-cyclic AMPs) suppressed B cell proliferation in a concentration-dependent manner and reversed the proliferation-enhancing effect of rolipram (Figure 5).

PDE4 activity is decreased in proliferating B cells

Increased PDE4 activity, either due to induction of gene expression or posttranslational modification, has been

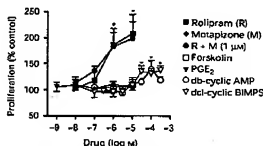


Figure 4 Influence of cyclic AMP elevating drugs on human B cell proliferation. B cells were plated on 96 well plates (5×10^5 cells in 200 μ L) and incubated with test compounds or the corresponding volume of DMSO (0.1% final concentration) for 30 min. Proliferation was induced by LPS (10 μ g mL^{-1}) plus IL-4 (100 U mL^{-1}) and determined at day 5 as described in legend to Figure 3. Values were calculated in relation to the proliferation signal of control incubations run in parallel (7550 \pm 1480 c.p.m., set at 100%). Data are mean values from 6–14 independent experiments; vertical lines show s.d. *Indicates statistical significance with $P<0.01$ compared to control.

Table 1 Cyclic AMP elevation by rolipram following mitogenic stimulation of human B lymphocytes

Treatment	Cyclic AMP (fmol/ 10^6 cells)
None	1086 \pm 612
LPS/IL-4	1051 \pm 390
LPS/IL-4 + rolipram	328 \pm 339*

CD19⁺ B cells were incubated for 1 h at 37°C in the presence of LPS (10 μ g mL^{-1})/IL-4 (100 U mL^{-1}). LPS/IL-4 + rolipram (10 μ M) or medium alone. Cells were washed twice, disrupted and cyclic AMP was extracted as described in the Methods section. Samples were stored at -20°C until cyclic AMP determination by EIA. Mean values \pm s.d. from 4–5 independent experiments are given. * $P<0.05$ versus other groups.

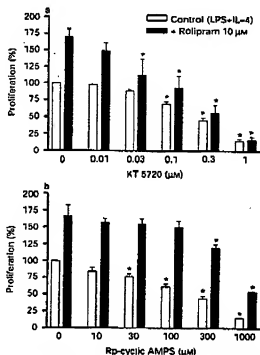


Figure 5 Influence of PKA inhibitors on human B cell proliferation. B cells were plated on 96 well plates (5×10^5 cells in 200 μ L) and incubated with PKA inhibitors (a) KT 5720 or (b) 8-Br-Rp-cyclic AMPs or the corresponding volume of DMSO (0.1% final concentration) for 30 min. Proliferation was induced by LPS (10 μ g mL^{-1}) plus IL-4 (100 U mL^{-1}) and determined at day 5 as described in legend to Figure 3. Values were calculated in relation to the proliferation signal of control incubations run in parallel (6850 \pm 1210 c.p.m., set at 100%). Data are mean values \pm s.d. from 4 (8-Br-Rp-cyclic AMPs) or 5 (KT 5720) independent experiments. *Indicates statistical significance with $P<0.01$ compared to the respective value in the absence of the PKA inhibitor.

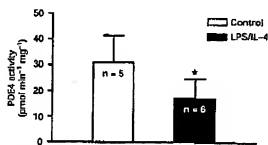


Figure 6 Decreased PDE4 activity in proliferating human B lymphocytes. B cells were either incubated with LPS ($10 \mu\text{g ml}^{-1}$) plus IL-4 (100 u ml^{-1}) or the same volume of medium as control. Five days later, cells were harvested and PDE4 activity was determined in the cytosolic fraction. Data are mean values \pm s.d.; n = number of individual determinations. *Indicates statistical significance with $P < 0.01$ compared to control.

repeatedly described in activated leucocytes (reviewed in Beavo, 1995). Thus, we wondered whether PDE4 activity would change upon induction of proliferation in human B lymphocytes. Five days following stimulation of CD19⁺ cells by LPS ($10 \mu\text{g ml}^{-1}$) and IL-4 (100 u ml^{-1}), PDE4 activity was significantly decreased compared to unstimulated control cells kept in culture for the same time (Figure 6). Messenger RNA analyses of two independent B cell samples revealed evidence for a 60% downregulation of the main PDE4 subtype, i.e. PDE4B2, in proliferating B cells at day 5, suggesting that the reduced PDE4 activity is due to a reduced expression of the corresponding genes).

Discussion

The present study was conducted to identify the PDE enzymes that are involved in the regulation of cyclic AMP levels in human B cells. This was of particular interest, since a variety of B cell responses are modulated by cyclic AMP (Holte *et al.*, 1988) and both stimulating and inhibiting effects (Simkin *et al.*, 1987; Anastassiou *et al.*, 1990; Roper & Phipps, 1992; Carrone & Banchereau, 1993; Coqueret *et al.*, 1996) on activation parameters of B lymphocytes have been attributed to the cyclic AMP/PKA signalling cascade.

The isolation method we used provided B cells of high purity without contamination by platelets or other blood cells. Similar to other leucocyte populations from peripheral human blood (summarized in Tenor & Schudt, 1996), the vast majority of the cyclic AMP hydrolyzing PDE activity of B lymphocytes was identified as soluble PDE4, presumably composed of the subtypes PDE4A, B and D, respectively (see Figures 1 and 2) as indicated by the detection of the corresponding mRNA transcripts. In addition, a significant amount of PDE7 activity was measured. Only very weak PDE3 activity was found in B cells, which is surprising, since human T lymphocytes contain equally high activities of PDE4 and PDE3 (Tenor *et al.*, 1994b; Giembycz *et al.*, 1996), both of which are functionally involved in the inhibitory effects of cyclic AMP in this cell type (Robiczek *et al.*, 1991; Essawy *et al.*, 1994; Banner *et al.*, 1995; Giembycz *et al.*, 1996). Thus, the human peripheral blood lymphocyte PDE profile differs substantially between the T and B lineage.

A direct comparison of B cells from normal and stope donors gave no evidence for an increased atopy-specific PDE4

activity, which is in agreement with recent PDE measurements performed in a variety of human leucocyte populations (Gantner *et al.*, 1997b). According to Hanfin and coworkers atopic monocytes, but not atopic lymphocytes, contain increased PDE4 activity (reviewed in Hanfin & Chan, 1995), which is in accordance with our present data.

Our functional studies with PDE inhibitors on activated B cells reflect the PDE activity pattern present in these cells. Whereas PDE3 inhibition was completely ineffective in any experimental system tested, PDE4 inhibition markedly affected B lymphocyte proliferation (see Figure 4). It should be mentioned that similar results were obtained in experiments in which the influence of cyclic AMP elevating drugs on anti-CD40 Ab/IL-4-induced release of IL-6 from human B cells was investigated. PDE3 inhibition was ineffective whereas PDE4 inhibition resulted in an increase of IL-6 release, although the effect was less pronounced compared to the stimulation of proliferation. Again, db-cyclic AMP mimicked the effect of PDE4 inhibitors up to $100 \mu\text{M}$, but neither PGE_2 nor forskolin significantly affected the release of IL-6 (data not shown).

In addition to the enhancing effect of PDE4 inhibitors on B cell growth, proliferating B cells displayed significantly lower PDE4 activity compared to resting cells (see Figure 6), most probably due to downregulation of PDE4 gene expression. This observation supports the concept that an increased cyclic AMP level due to diminished PDE4 activity promotes B lymphocyte proliferation under our experimental conditions. Two lines of evidence support the hypothesis of cyclic AMP as a critical regulator of B cell growth: first, the increased proliferative response induced by rolipram is paralleled by a rise in cyclic AMP and, secondly, PKA inhibitors not only abolished the PDE4 inhibitor effects as expected, but also strongly inhibited LPS/IL-4-induced DNA synthesis of control cells (see Figure 5). This suggests that a cyclic AMP signal is necessary to allow the initiation of DNA synthesis in human B lymphocytes.

Interestingly, the different PKA inhibitors tested behaved oppositely on control cells and on cells conubated with rolipram. Whereas KT 5720 more potentially affected the proliferation in the presence of rolipram, 8-Br-Rp-cyclic AMPs more potently abrogated DNA synthesis under control conditions (see Figure 5). These effects imply a different mechanistic mode of PKA inhibition by these two agents.

PDE4 inhibitors are known to suppress a wide variety of inflammatory cell functions (reviewed in Dent & Giembycz, 1996) among which the antiproliferative effects on T lymphocytes have been extensively studied. In this respect, it was interesting to find an enhancement of polyclonal B cell proliferation in the presence of PDE4 inhibitors under our experimental conditions, emphasizing basic differences in the PDE profile and in PDE function between B and T lymphocytes. In addition, it is important to point out that earlier studies provided conclusive evidence for growth inhibiting effects by cyclic AMP elevating drugs on B cells (reviewed in Sanders, 1995). This suggests that a certain response triggered via the cyclic AMP/PKA pathway in B lymphocytes may be manifoldly influenced. These cyclic AMP signal modulators may include (i) the manner of stimulation, i.e. the nature of the B cell stimulus, (ii) the signal intensity and the duration of the cyclic AMP increase, (iii) the time point of measurement of a certain response, and (iv) the actual activation/differentiation status of the B cells under investigation.

For instance, it has been shown that β -adrenoceptor-mediated elevation of cyclic AMP inhibits the proliferation induced by LPS or anti-immunoglobulin antibodies. In contrast, activation of the same receptor enhances proliferation of B cells exposed to PMA/ionomycin (reviewed in

Sanders, 1995). Similar effects have been described for cholera toxin (CT), which leads to a cyclic AMP increase via activation of adenylate cyclase. Whereas CT stimulated anti-IL-2-induced B cell proliferation, CT selectively inhibited IL-2- but not IL-4-induced DNA synthesis (Garrone & Banchereau, 1993).

Our own data point to the importance of the cyclic AMP signal intensity in directing the fate of B cells. In accordance with experiments on human tonsillar B lymphocytes (Garrone & Banchereau, 1993), concentrations of db-cyclic AMP up to 100 μ M increased polyclonally-induced DNA synthesis, whereas 1 mM of the same compound resulted in a strong inhibition of proliferation, presumably by the induction of cytotoxicity. The fact that mM concentrations of db-cyclic AMP have been shown to shorten the life span of human resting peripheral blood B cells *in vitro* by accelerating the onset of apoptosis (Lemo et al., 1995), reveals a mechanistic explanation for the latter observation and supports the concept of a bimodal cyclic AMP action on the control of B cell growth.

Furthermore, the time point of measuring the proliferative response might be critical. Data illustrated in the paper by Garrone and Banchereau (1993) showed that the kinetics of IL-4-dependent proliferation were significantly modified by agents increasing cyclic AMP, since CT, PGE₂ and forskolin partially inhibited the early [H]-thymidine uptake induced by IL-4 within 48 h, but strongly stimulated it when monitored after 3 or more days of culture. Thus, our data obtained with the PDE4 inhibitor rolipram and piclamilast are in accordance with this finding, since we investigated the role of cyclic AMP in the regulation of B lymphocyte proliferation in a 5 day experiment using the same cosimulus (IL-4).

Finally, the effect of cyclic AMP on the regulation of proliferation may depend on the activation or differentiation level of B cells. For instance, cyclic AMP elevating agents were shown to increase synergistically DNA synthesis of B cells exposed to low-mitogenic anti-IgM B preparations, but did not enhance proliferation of B cells that were costimulated or pre-

activated with mitogenic concentrations of the superantigen *Staphylococcus aureus*, strain cowan I (Anastasiou et al., 1992).

Interestingly, PDE4 inhibitors were far more efficient than cyclic AMP analogues in enhancing B cell growth, although no other mechanism apart from elevation of cyclic AMP through the inhibition of its breakdown is known for PDE4 inhibitors. A theoretical explanation could be specific subcellular compartmentalization of PDE4 and PDE4 inhibitors at sites where a local increase in cyclic AMP triggers a proliferation enhancing signal. Since the use of cyclic AMP analogues always results in a net effect integrated over the whole cell, the results might differ not only quantitatively, but also qualitatively from the ones obtained with PDE4 inhibitors.

Surprisingly, we failed to find any effects of PGE₂ or forskolin on LPS/IL-4-induced proliferation, although other investigators have shown modulatory effects of both agents in human B cell systems (Garrone & Banchereau, 1993) and B cells, at least in the murine system (Fedyk et al., 1996), functionally coexpress a variety of prostaglandin receptors including the Gs-coupled EP₁ and EP₂ type.

In summary, the present paper describes the detailed PDE activity and mRNA expression pattern of human peripheral blood B lymphocytes, which substantially differs from the PDE profile of T lymphocytes, and underlines the prominent role of the PDE4 family in leukocytes. The direct comparison of normal and atopic cells reveals no evidence for a PDE dysregulation in B lymphocytes due to the atopy status. Furthermore, our data indicate that cyclic AMP/PKA-dependent signalling pathways are involved in the proliferative response of human B lymphocytes following stimulation with LPS/IL-4.

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PDE7A is expressed in human B-lymphocytes and is up-regulated by elevation of intracellular cAMPRichard Lee^a, Sharon Wolda^b, Eunyi Moon^a, James Esselstyn^b,
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Abstract

PDE7A is a recently described 3',5'-cyclic adenosine monophosphate (cAMP)-specific phosphodiesterase (PDE) whose expression has been detected in T-cells. As treatment with the methylxanthine theophylline, a nonspecific PDE inhibitor, induces apoptosis in leukemic cells from patients with the B-lineage malignancy chronic lymphocytic leukemia (CLL), we sought to determine if PDE7A was a target of theophylline therapy in such cells. Western analysis revealed expression of PDE7A in normal human splenic B-cells, primary CLL cells, and in a CLL-derived cell line (WSU-CLL). Among the six cAMP PDEs (PDE1B, PDE3B, PDE4A, PDE4B, PDE4D, and PDE7) examined in WSU-CLL, only PDE7A levels were augmented by treatment with methylxanthines. The activity of PDE7A isolated from the WSU-CLL cell line by immunoprecipitation was inhibited by theophylline and IBMX with IC₅₀ values of 343.5 and 8.6 μM, respectively. WSU-CLL PDE7A was also up-regulated by a novel specific inhibitor (IC242), which inhibits PDE7A from WSU-CLL cells with an IC₅₀ value of 0.84 μM. IC242-mediated up-regulation of PDE7A was blocked by the protein kinase A (PKA) inhibitor H-89. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: PDE7; Cyclic nucleotide phosphodiesterases; Theophylline; CLL; cAMP**1. Introduction**

Elevated intracellular levels of 3',5'-cyclic adenosine monophosphate (cAMP) can induce apoptosis in susceptible subpopulations of both B- and T-lineage lymphocytes. This suggests that agents capable of modulating cAMP levels might be useful for the treatment of lymphoid malignancies [1]. One means of augmenting cAMP signaling has been through the use of cAMP phosphodiesterase (PDE) inhibitors, as inhibition of cAMP catabolism results in elevation of intracellular lymphoid cAMP levels in vivo [2]. Theophylline, a non-specific methylxanthine PDE inhibitor, has been shown to induce apoptosis in chronic lymphocytic leukemia (CLL) B-lymphocytes in vitro [3,4]. A subsequent Phase 2

clinical trial demonstrated that combined treatment with theophylline and chlorambucil induced positive responses in CLL patients who failed treatment with chlorambucil alone [5]. More recently, Makower et al. [6] have reported responses to theophylline monotherapy in three patients with advanced CLL.

Theophylline is a nonselective PDE inhibitor as well as an adenosine receptor antagonist, complicating both the clinical and research applications of this reagent. A more selective PDE inhibitor might also induce apoptosis in lymphoid cells and have therapeutic value in the treatment of lymphoid malignancies. Lymphoid cells contain several classes of cyclic nucleotide PDEs, including cGMP-inhibited PDE3 [7] and cAMP-specific PDE4 [8]. Inhibition of PDE4 with rolipram, an inhibitor specific for this class of enzymes, induces apoptosis of lymphocytes isolated from CLL patients [9]. Peripheral T-cells are resistant to rolipram-induced apoptosis and the susceptibility of CLL cells and T-cells to PDE4 inhibitors correlated with their sensitivity to apoptosis induced by the cell-permeable cAMP analog dibutyryl cAMP (dbcAMP) [9].

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Since the initiation of our work on PDEs and CLL, interest has grown in the potential role of a novel cAMP PDE, PDE7A, in lymphoid cyclic nucleotide metabolism. PDE7A was originally cloned by Michaeli et al. [10] as a result of its ability to complement the heat-shock-sensitive phenotype of a *Saccharomyces cerevisiae* strain in which two yeast cAMP PDE genes had been disrupted. There are two splice variants of the PDE7A gene, encoding enzymes with differing sequence and tissue distribution. PDE7A1, which contains a unique hydrophilic 45 residue NH₂-terminus sequence, has been detected in lymphoid cells and migrates with an apparent MW of 55 kDa [11]. In contrast, PDE7A2, which contains a unique hydrophobic 20 residue N-terminus sequence, was cloned from skeletal muscle cDNA libraries and migrates with an apparent MW of 50 kDa [12]. A second member of the PDE7 gene family, PDE7B, was recently cloned [13–15]. Expression of PDE7B was detected in multiple tissues, but not in lymphoid cells.

PDE7A1 has been detected in both primary T-cells and cell lines derived from T-cells [11,16]. PDE7A is reportedly up-regulated in T-cells upon costimulation with CD3 and CD28 and is required for T-cell proliferation [17]. The expression of PDE7A in B-lymphocytes is not as well documented. Gantner et al. [18] reported substantial PDE activity in primary human B-cells that were not inhibited by inhibitors specific for PDE1, PDE3, or PDE4. This residual cAMP-hydrolyzing activity was attributed to PDE7. Li et al. [17] examined the expression of PDE7A in two human B-cell lines, Jijoye and Ramos. While PDE7A transcript was detected in the B-cell lines, no PDE7A protein was detected in these cells by Western analysis.

The goal of the current study was to determine whether PDE7A is expressed in CLL B-lymphocytes and to assess whether the activity of this enzyme can be inhibited by methylxanthines. In this report, we demonstrate that PDE7A is expressed in normal B-cells, primary CLL cells, and the CLL B-cell line WSU-CLL. Furthermore, we demonstrate that PDE7A protein expression is up-regulated in WSU-CLL cells in response to treatment with theophylline and IBMX, suggesting a feedback mechanism that enables additional control of cellular cAMP levels. Lastly, we demonstrate similar changes in PDE7 protein expression in response to treatment with a selective PDE7A inhibitor.

2. Methods

2.1. Reagents

DPCPX and rolipram were from RBI (Natick, MA). Forskolin and IBMX were from Sigma (St. Louis, MO). Theophylline was obtained as a 3.2-mg/ml solution in 5% dextrose (Baxter Healthcare, Deerfield, IL). H-89 was from Calbiochem (San Diego, CA). Compounds IC242 and IC243 were obtained from ICOS, Bothell, WA.

2.2. Cell purification and culture

WSU-CLL cells were generously provided by Dr. R. Mohamed (Wayne State University). Primary leukemic cells were isolated from the peripheral blood of patients with acute T-cell leukemia (ATL) or CLL after obtaining IRB-approved informed consent. The diagnosis of each patient's malignancy was confirmed by characteristic immunophenotype. Primary splenic B-cells were isolated from the discarded splenic tissue of a patient who underwent splenectomy for idiopathic thrombocytopenic purpura. Primary peripheral blood T-cells were obtained from Leucopaks. Normal or leukemic cells or WSU-CLL cells were grown in RPMI supplemented with 10% fetal bovine serum, 2 mM/L-glutamine, 1% penicillin/streptomycin (Sigma), 10 mM/Hepes, pH 7.4.

2.3. Antibodies

Monoclonal antibodies directed against PDE1B, PDE3B, PDE4A, PDE4B, and PDE7A were generated using fusion proteins derived from the glutathione S-transferase (GST) expression system (Pharmacia). The PDE1B antibody 114D was generated from a GST fusion protein with cDNA corresponding to amino acids 457–536 of PDE1B [19]. The PDE3B antibody 281K was generated from a GST fusion protein with cDNA corresponding to amino acids 520–879 of PDE3B [20]. The PDE4A antibody 66C12H was generated from a GST fusion protein with cDNA corresponding to amino acids 718–886 of PDE4A3 [21]. The PDE7A1-specific antibodies 144N and 144R were generated from a GST fusion protein with cDNA corresponding to amino acids 18–190 of PDE7A1 [10]. The GST fusion constructs described above were transformed into the *Escherichia coli* strain XL-1 Blue (Stratagene) and protein expression was induced with isopropyl- β -D-thiogalactoside (IPTG) as described by the manufacturer (Pharmacia). The expressed fusion proteins were isolated from bacterial inclusion bodies by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electroelution. The PDE4B monoclonal antibody 96G7A was generated using full-length PDE4B2 expressed in and purified from *E. coli* (Lothar Uher and Vince Florio, unpublished data) [21]. A PDE7A pan-reactive antibody (184O) that detects both PDE7A1 and PDE7A2 was generated from gel-purified, full-length PDE7A1 expressed in *E. coli*. Monoclonal antibodies selective for the appropriate PDEs were generated in mice using standard procedures [22]. Each antibody was tested by Western analysis for its selective reactivity against the respective full-length recombinant PDE from which it was derived. Each antibody was also tested by Western analysis for its lack of reactivity with other PDE family members. Each of the monoclonal antibodies described (with the exception of 281K) was purified from mouse ascites by Protein-A chromatography. The source of the monoclonal antibody 281K was a hybridoma culture supernatant. The

antibody against PDE4D (61D10E) has been previously described [23].

2.4. Protein extraction and Western blot analysis

Cells were washed once with ice-cold PBS. After centrifugation at 4000 rpm for 5 min (relative centrifugal force [RCF] = 1310), the cell pellet was lysed for 20 min in lysis buffer: 50 mM Tris pH 7.4; 1% NP-40; 125 mM NaCl; 2 µg/ml aprotinin, leupeptin, and pepstatin; 1 mM PMSF; 1 mM NaF. The protein supernatant was collected after centrifugation at 14,000 rpm (RCF = 16,000) at 4 °C for 5 min. Protein concentration was measured with BCA reagents (Pierce, Rockford, IL). The proteins were separated on 7.5% or 10% SDS-PAGE using 10–30 µg protein per lane and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked in 5% skim milk in PBS/0.05% Tween 20 (PBS/T) at room temperature for 1 h. Anti-PDE or anti-tubulin antibodies (1 µg/ml final) were added in blocking buffer and incubated at room temperature for 1 h with shaking. The membrane was washed four times each for 5 min with PBS/T. HRP-conjugated antiserum IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was then added (1:1000 dilution), incubated, and washed in a manner identical to that described for the first antibody. Immunoreactive protein was then detected by the ECL technique according to the vendor's protocol (Pierce).

2.5. RT-PCR and Northern analysis

RNA was isolated from primary CLL or WSU-CLL cells using Ultraspec reagent (Biotec, Houston, TX). cDNA was synthesized from 10 µg of total RNA using oligo d(T) primers and Moloney murine leukemia virus reverse transcriptase in a final volume of 40 µl (Stratagene, La Jolla, CA). One microliter of the first strand cDNA product was then used as template for PCR amplification with Taq DNA polymerase (Gibco BRL, Frederick, MD) by denaturing the template for 4 min at 94 °C, followed by 40 thermocycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min. For generation of a 403-bp probe homologous to sequence common to both PDE7A1 and PDE7A2, we used the following oligonucleotides: sense GGAAATAGTCTAGTAAGCTTAAC (first 549 bp); antisense GGCAGATGTGAGAAATAAGCCTG. The PCR product was subcloned into a plasmid vector (pCR11, Invitrogen, Carlsbad, CA) and subsequently utilized for PCR-based amplification of α³²P dCTP-labeled probes for hybridization to Northern blots.

2.6. cAMP assay

The cAMP assay was performed as previously described, with the exception that samples were processed using the acetylation protocol according to the manufacturer's recommendations (NEN, Boston, MA) [9].

2.7. PDE activity assays

The inhibition of human PDE enzyme activity by IC242 and IC243 was determined using a standard PDE assay as described below and recombinant protein expressed in yeast or baculovirus-infected *Spodoptera frugiperda* sf9 cells. PDE1B, PDE2A, PDE4B, and PDE5 were expressed and purified from a *S. cerevisiae* strain lacking endogenous PDE activity. PDE8, 9, and 10 were also expressed in yeast and were used in the assay as total cell extracts. PDE7 was expressed in yeast and used in the assay as a soluble extract. PDE1A and PDE1C were expressed and purified from baculovirus-infected sf9 cells. PDE3A was expressed in baculovirus-infected sf9 cells and used in the assay as a particulate membrane fraction. cAMP was used as the substrate for assays with PDE1C, PDE3A, PDE4B, PDE7A, PDE8, and PDE10 while cGMP was used as the substrate for assays with PDE1A, PDE1B, PDE2A, PDE5A, and PDE9.

PDE assays were performed using the Biomek 1000 robotic workstation (Beckman Instruments). Assays were performed in PDE assay buffer (40 mM Tris-Cl, pH 8.0, 1 µM ZnSO₄, 5 mM MgCl₂; 0.1 mg/ml bovine serum albumin (BSA), 0.4 µg/ml bovine calmodulin, and 0.2 mM CaCl₂), using either [³²P]cAMP or [³²P]cGMP [specific activity of 25 Ci/mmol (ICN Biomedicals)] at a final concentration of 32 nM. At this substrate concentration, the observed IC₅₀ value closely approximates the inhibition constant (K_i) for each enzyme. The inhibitors IC242 and IC243 were tested over a concentration range of 0.005–100 µM. The reaction was initiated by addition of PDE enzyme at a submaximal concentration previously determined to hydrolyze 20–50% of the cyclic nucleotide in the absence of inhibitor. The assay was run for 12 min at 30 °C followed by addition of *Crotalus atrox* snake venom (15 mg/ml in 0.01 M Tris-Cl, pH 8.0). The reaction was terminated by addition of activated charcoal (25 mg/ml in 0.1 M NaH₂PO₄) to bind unincorporated phosphate. The supernatant was removed and hydrolysis quantitated by Cerenkov counting. Dose-response curves were fitted using a four-parameter logistic model described by the equation $y = a + b / (1 + (x/c)^d)$ where y is the total percent PDE activity, a is PDE activity at infinite inhibitor (Y_{max}), b is the net PDE activity at zero inhibitor (Y_{min}), c is the inhibitor concentration at 50% maximal activity (IC₅₀), x is the inhibitor concentration, and d is the slope of the curve at the IC₅₀ (Hill coefficient).

2.8. Immunoprecipitation of PDE7 and PDE assay

WSU-CLL cells were suspended (1×10^6 cells/1.25 ml) in BTP buffer (50 mM bis-tris propane, pH 7.2, 1 mM dithiothreitol [DTT], 5 mM EGTA, 1 mM EDTA, 0.5 mM AERSF, and 5 µg/ml each of pepstatin, leupeptin, aprotinin) and homogenized with 100 strokes using a tight dounce. The cell homogenate was centrifuged at 44,000 rpm for 1 h.

The supernatant fraction was used for immunoprecipitation. Pansorbin beads (Calbiochem) were loaded with rabbit antiserum antibody (Pierce) and washed once with PBS/1% BSA. On the day of the experiment, the preloaded beads were incubated with the PDE7A1-specific monoclonal antibody 144R (30 μ g/30 μ l of slurry) at 4 °C for 1 h. The sample was spun at 2000 rpm for 5 min. The supernatant was saved for assay. The beads were washed twice in PBS/1% BSA, resuspended in PBS/1% BSA, and used directly for PDE assays. PDE activity was measured as described above directly on the immunoprecipitated PDE7A-Pansorbin beads in a buffer containing 45 mM Tris-Cl, pH 8.0, 20 mM NaCl, 2 μ M ZnSO₄, 6 mM MgCl₂, 0.12 mg/ml of BSA, and 0.02 mM DTT for 15 min at 30 °C [21]. The [³²P]cAMP substrate concentration was 0.16 μ M. For the inhibitor IC₅₀ determinations, the inhibitors IBMX, theophylline, and IC242 were tested over a concentration range of 0.015–300 μ M. Dose-response curves were fitted using the four-parameter logistic model as described above. Dose-response curves for theophylline were fitted using a three-parameter logistic model described by the equation $y = a/(1 + (x/b)^c)$ where y is the total percent PDE activity, a is the net PDE activity at zero inhibitor (Y_{max}), b is the inhibitor concentration at 50% maximal activity (IC₅₀), x is the inhibitor concentration, and c is the slope of the curve at the IC₅₀ (Hill coefficient). In the three-parameter model, the percent inhibition at infinite inhibitor (Y_{min}) is fixed at zero. This was done because of the poor potency and solubility of theophylline and allows for a more accurate determination of the IC₅₀. The r^2 correlation coefficient values for the IBMX, theophylline, and IC242 curves are .999, .999, and .999, respectively, as determined by TableCurve 2D (SPSS, AISN Software).

3. Results

We initially sought to determine whether PDE7A is expressed in human lymphoid cells from patients with CLL. Western analysis was used to assess the expression of PDE7A in isolated splenic B-cells, T-cells, and lymphoid cells from patients with either ATL or CLL (Fig. 1A). PDE7A was readily detected in isolated primary splenic B-cells as well as primary peripheral blood T-cells isolated from healthy individuals. In addition, PDE7A was readily detected in lymphoid cells from a CLL patient. In contrast, a PDE7A immunoreactive band was not detected in lymphoid cells from a patient with ATL. We then went on to characterize PDE7 expression in a B-cell line (WSU-CLL) derived from the peripheral blood of a patient with CLL [24]. For this experiment, WSU-CLL cells were treated for 8 h with IBMX (a nonselective PDE inhibitor), forskolin, dbcAMP, or the PDE4 inhibitor rolipram and then analyzed by Western analysis with a PDE7A-specific antibody (Fig. 1B). PDE7A was detectable at low levels in untreated WSU-CLL but was significantly increased after

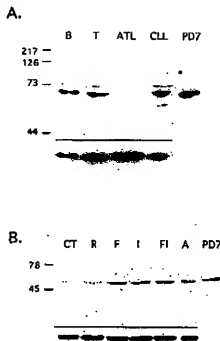


Fig. 1. PDE7A expression in primary human lymphoid cells and the CLL cell line, WSU-CLL. Panel A: Whole cell lysates (30 μ g) from normal human splenic B-lymphocytes (B), peripheral blood T-cells (T), ATL cells (ATL), and CLL cells (CLL) were analyzed by Western blot using a PDE7A1-specific monoclonal antibody. Recombinant PDE7A1 was also run as a control (PD7). Panel B: A similar analysis was performed on whole cell lysates (10 μ g) from the CLL cell line, WSU-CLL. WSU-CLL cells were exposed for 8 h to media (CT), 10 μ M rolipram (R), 40 μ M forskolin (F), 50 μ M/ml IBMX (I), both forskolin and IBMX (FI), or 100 μ M dbcAMP (A). Equal loading was verified by blotting for tubulin, as shown in the panel below each immunoblot.

8 h of treatment with 50 μ M/ml IBMX. Forskolin and dbcAMP were also able to increase PDE7A expression relative to untreated cells. IBMX does not appear to be elevating PDE7 levels by nonselective inhibition of PDE4 activity because incubation of WSU-CLL cells with the potent PDE4 inhibitor rolipram at 10 μ M had no effect on PDE7 expression (Fig. 1B, Lane R). Similar studies using the PDE3 inhibitor cilostamide (10 μ M) also failed to up-regulate PDE7A levels (data not shown).

Additional experiments were done to determine whether PDE inhibitors and cAMP elevating agents, alone or in combination, could alter the expression of other PDE isoforms in the WSU-CLL cells (Fig. 2). Cells were treated for 8 h as described above and then prepared for Western analysis. PDE1B was not detected in WSU-CLL under any of the test conditions. PDE3B was constitutively expressed in WSU-CLL and modestly up-regulated in response to forskolin and dbcAMP. IBMX in combination with forskolin did not further potentiate the level of PDE3B expression. PDE4A, B, and D were not detected in untreated

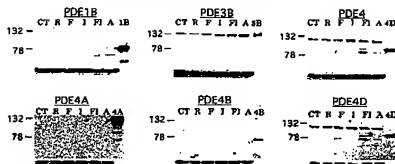


Fig. 2. Regulation of PDE7 expression in WSU-CLL. Thirty micrograms whole cell lysate protein was analyzed by Western blot analysis using monoclonal antibodies specific for PDE1B, PDE3B, PDE4, PDE4A, PDE4B, and PDE4D as indicated. The PDE4 (96F26) antibody was "pan-reactive," i.e., raised against a peptide common to all known PDE4 gene family members. Treatment with media or drugs is as indicated in the legend for Fig. 1. Recombinant PDEs were run in the right-hand lane of each Western as a positive control. Equal loading was verified by blotting for tubulin, as shown in the panel below each immunoblot.

WSU-CLL cells. Treatment with forskolin and dbcAMP induced weak expression of a 74-kDa immunoreactive band detected by the PDE4 pan-reactive antibody (96F26) and the PDE4D-specific antibody (61D10E). The combination of forskolin and IBMX further increased the expression of the 74-kDa band as well as a band of 80 kDa. IBMX alone had no effect on PDE4D expression. A band at 115 kDa detected with both the pan-reactive and the PDE4D-specific antibody was unaltered by the various treatments.

The results described above suggest that PDE7A is up-regulated in response to IBMX in the absence of exogenous activation of adenylate cyclase. A dose titration was done to investigate the concentration of IBMX necessary to induce this up-regulation (Fig. 3A). A dose as low as 20 μ M (90 μ M) was able to significantly increase the expression of PDE7A after 8 h of treatment. Increases in PDE7 activity were also noted in response to IBMX treatment. For this experiment, PDE7A was immunoprecipitated from treated or untreated WSU-CLL and associated cAMP-hydrolyzing

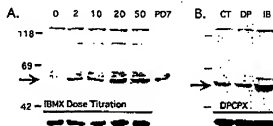


Fig. 3. Analysis of IBMX-induced up-regulation of PDE7A. Panel A: WSU-CLL cells were exposed for 8 h to the indicated concentration of IBMX (μ M), then analyzed by Western blot using monoclonal antibodies specific for PDE7A. Recombinant PDE7A was included as a positive control. Panel B: WSU-CLL cells were cultured for 8 h in media (CT), the adenosine receptor antagonist DPCPX (DP: 1 μ M), or 50 μ M IBMX (IB).

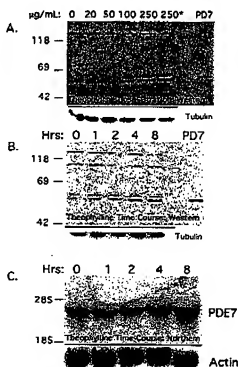


Fig. 4. Analysis of theophylline-induced up-regulation of PDE7A. Panel A: WSU-CLL cells were exposed for 8 h to the indicated concentration of theophylline (μ M/ml). The sample indicated with an asterisk was incubated for 20 h. Panel B: WSU-CLL cells were exposed for the indicated number of hours to 250 μ M/ml theophylline. Panel C: WSU-CLL cells were exposed to 250 μ M/ml theophylline for the indicated number of hours. RNA isolated from the cells was then analyzed by Northern analysis using a 32 P-labeled nucleic acid probe specific for sequence common to PDE7A1 and PDE7A2 transcripts. Equal RNA loading and transfer was verified by stripping the Northern and reprobing with an actin-specific nucleic acid probe (lower panel).

activity was assayed as described in the Methods section. PDE7A-associated PDE activity increased 4.8-fold in IBMX-treated (18 h) WSU-CLL cells ($n=3$; mean activity 4.01 ± 0.54 pmol/min/mg total protein) relative to untreated controls ($n=3$; mean activity 0.79 ± 0.20 pmol/min/mg total protein).

Given that methylxanthines are known to have activity both as nonspecific PDE inhibitors and as adenosine receptor antagonists, we determined whether an adenosine receptor antagonist without PDE inhibitory activity could increase PDE7A expression. An 8-h treatment of WSU-CLL cells with DPCPX at $1 \mu\text{M}$ (IC₅₀ for A₁ receptors = 0.69 nM ; A_{2a} receptors = 502 nM ; A_{2b} < 100 nM) had no effect on PDE7A expression (Fig. 3B) [25].

As theophylline is currently being tested for treatment of CLL, we investigated whether this methylxanthine derivative could also increase PDE7A expression in the WSU-CLL cell line. Western analysis with a PDE7A selective antibody was used to look at changes in PDE7A expression in response to increasing concentrations of theophylline (Fig. 4A). As was noted for IBMX, theophylline was able to increase PDE7A protein expression in a dose-dependent manner with a significant increase in expression at theophylline concentrations of $100 \mu\text{g/ml}$ ($550 \mu\text{M}$) and higher. Western analysis was then used to determine the time course of this effect (Fig. 4B). WSU-CLL cells were treated with $100 \mu\text{g/ml}$ theophylline and harvested 1–8 h after the initiation of treatment. An immunoreactive band of 55 kDa consistent with PDE7A was detected as early as 4 h after treatment with theophylline. Lastly, Northern analysis was used to look at possible changes in PDE7 transcript levels in response to theophylline over the same time course (Fig. 4C). For this experiment, a fragment of PDE7A (549–958 bp) was cloned by PCR from CLL cDNA as described in the Methods section. This fragment was used as a probe for Northern analysis of RNA isolated from WSU-CLL cells

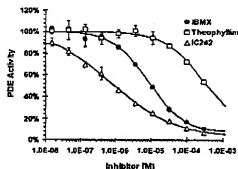


Fig. 5. Inhibition of WSU-CLL PDE7A by IBMX, theophylline, and IC242. PDE7 was immunoprecipitated from WSU-CLL cells and its activity measured in the presence of concentrations of IBMX, theophylline, and IC242 ranging from 0.015 to $300 \mu\text{M}$. The SEM of triplicate determinations is shown.

Table 1
Inhibition of human PDE activity by IC242 and IC243

PDE enzyme	IC ₅₀ (μM)	
	IC242	IC243
1A	115	15
1B	203	92
1C	41	58
2A	726	650
3A	111	47
4B	72	48
5A	283	21
7A	0.37	43
8A	36	78
9A	998	515
10A	35	3.3

The values shown are IC₅₀ values (μM).

treated with $250 \mu\text{M}$ theophylline over an 8-h time course. The PDE7A transcript of $\sim 4 \text{ kb}$ was detected at relatively high levels in untreated WSU-CLL cells and was not significantly altered in response to 8 h of treatment with theophylline.

We next looked at the ability of IBMX and theophylline to directly inhibit PDE7 activity in WSU-CLL cells. For these experiments, PDE7 was immunoprecipitated from WSU-CLL cells using a PDE7 selective antibody and the activity of the purified enzyme was measured in the presence of varying concentrations of these inhibitors. As shown in Fig. 5, IBMX and theophylline inhibit PDE7 activity derived from WSU-CLL cells with IC₅₀ values of 8.6 and $343 \mu\text{M}$, respectively. Thus, the concentrations of IBMX ($20 \mu\text{g/ml} = 90 \mu\text{M}$) and theophylline ($100 \mu\text{g/ml} = 550$), which augmented PDE7A levels in WSU-CLL cells, were above the IC₅₀ values of these compounds for PDE7, raising

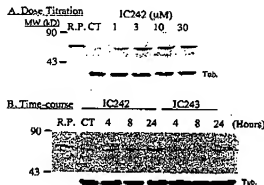


Fig. 6. IC242 augments PDE7A levels in WSU-CLL cells. Panel A: WSU-CLL cells were incubated for 18 h in the indicated concentrations of IC242, followed by lysis and immunoblotting for PDE7A. Panel B: WSU-CLL cells were incubated for the indicated number of hours in $10 \mu\text{M}$ IC242 or IC243, followed by lysis and immunoblotting for PDE7A. R.P. indicates recombinant PDE7A used as a control. As shown in the panels below each immunoblot, equal loading of lanes was verified by subsequent blotting for tubulin (Tub.).

the possibility that the observed increases in PDE7 resulted from direct inhibition of this enzyme.

To test this, we used a specific PDE7 inhibitor, IC242, to examine changes in PDE7 levels. IC242 inhibits recombinant PDE7 with an IC_{50} value of 0.37 μ M and is 100-fold selective for PDE7 relative to other recombinant PDEs (Table 1). IC242 inhibited PDE7 immunoprecipitated from WSU-CLL cells with an IC_{50} of 0.84 μ M (Fig. 5). Treatment of WSU-CLL cells with a range of IC242 concentrations for 18 h resulted in significant increase in PDE7A expression at concentrations above 3 μ M (Fig. 6A). A time course experiment demonstrated that PDE7A was up-regulated within 8 h of exposure to 10 μ M IC242 (Fig. 6B). Because IC242 is a

steroid-like compound, we used a structurally related steroid (IC243) that has no PDE7 activity as a negative control (Table 1). IC243 at 10 μ M has no effect on PDE7A expression over a 24-h time course (Fig. 6B). These results suggest that IC242 is a potent inhibitor of PDE7A that can induce increases in PDE7A expression comparable to what was observed with IBMX and theophylline.

We finally looked at changes in cAMP levels in WSU-CLL cells in response to IBMX and IC242 (Fig. 7). Cells were incubated with increasing concentrations of IBMX from 0 to 250 μ g/ml for 1 h and then assayed for cAMP content. The cAMP level increased in response to increasing concentration of IBMX up to 4.5-fold above baseline (Fig. 7A). In contrast, incubation of WSU-CLL cells with increasing concentrations of IC242 had no measurable effect on cAMP levels, even at doses previously shown to increase the expression of PDE7A (Fig. 7B). In order to examine the possibility that the absence of a rise in cAMP following IC242 treatment is the result of concomitant inhibition of adenylyl cyclase by this drug, we cotreated WSU-CLL cells with both IBMX and IC242. cAMP levels rose comparably in WSU-CLL cells treated with either IBMX alone or IBMX combined with 10 μ M IC242 (data not shown). Thus, we found no evidence to suggest that IC242 inhibits adenylyl cyclase. As many cAMP-mediated signaling events result from activation of protein kinase A (PKA), we examined whether the IC242-mediated up-regulation of PDE7A expression could be inhibited by cotreatment of cells with 500 nM H-89, a potent and selective isquinolinesulfonamide inhibitor of PKA (IC_{50} =48 nM) [26]. H-89 blocked IC242-mediated up-regulation of PDE7A, suggesting that despite the absence of detectable changes in total cellular cAMP, IC242 exerts its effect by a cAMP and PKA-dependent pathway (Fig. 7C).

4. Discussion

We find constitutive expression of PDE7A in human splenic B-cells, peripheral blood T-cells, and in lymphoid cells from patients with CLL. In a CLL cell line, WSU-CLL, PDE7A expression is up-regulated by PDE inhibitors, such as theophylline and IBMX, as well as adenylyl cyclase activators such as forskolin and the cAMP analogue, dbcAMP. As each of these stimuli raise cAMP levels in WSU-CLL cells (Fig. 7 and data not shown), this would suggest that the observed up-regulation of PDE7A is a compensatory feedback loop that augments the cell's ability to catabolize cAMP in the face of increased intracellular cAMP levels. Similar compensatory increases in PDE levels have been observed for PDE4 [27]. As an example, both transcriptional (PDE4D) and posttranscriptional (PDE4B) mechanisms result in follicle-stimulating hormone up-regulation of PDE4 species in rat Sertoli cells [28]. In the case of WSU-CLL, we have found

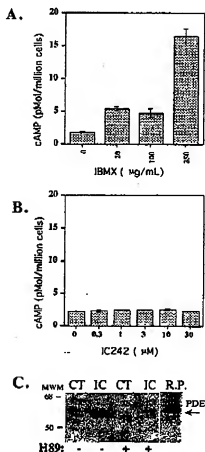


Fig. 7. Role of cAMP in IC242-mediated upregulation of PDE7 levels. Panel A: Samples of one million WSU-CLL cells were incubated in media or the indicated concentration of IBMX for 1 h, prior to lysis and determination of cAMP concentration by RIA. The SEM of triplicate samples is indicated. Panel B: One million WSU-CLL cells were treated with the indicated concentration of IC242 for 25 min, followed by determination of cAMP levels. Panel C: WSU-CLL cells were treated with media alone (CT) or 10 μ M IC242 (IC) for 8 h in the presence (+) or absence (-) of 500 nM H-89. Cells were then lysed and immunoblotted for PDE7A. R.P. indicates recombinant PDE7A used as a control.

that basal PDE7A transcript levels are high, suggesting that at least a part of the observed up-regulation may be translational or posttranslational.

As at least some of the clinical effects of methylxanthines are due to their effects on lymphoid cells, we wished to determine whether lymphoid PDE7A was efficiently inhibited by such drugs. IBMX inhibited PDE7A with an IC_{50} value of 8.6 μ M. In contrast, the clinically useful compound theophylline was a relatively poor inhibitor of PDE7A (IC_{50} = 343.5 μ M). Given that therapeutic concentrations of theophylline (5–20 μ g/ml, corresponding to 27.5–110 μ M) are well below the IC_{50} value for PDE7A, it appears unlikely that PDE7A is a relevant therapeutic target in the clinical use of this compound.

As with the nonselective methylxanthines, IC242 also induced up-regulation of PDE7A in WSU-CLL cells. In contrast to these drugs, however, IC242 failed to increase WSU-CLL cAMP levels. Although it is formally possible that IC242 may up-regulate PDE7A by a cAMP-independent mechanism, we favor the alternative hypothesis that augmentation of PDE7A by IC242 is driven by cAMP-mediated signal transduction in a subcellular compartment that does not contribute significantly to total cellular cAMP levels. Consistent with this idea, we find that IC242-mediated augmentation of PDE7A is blocked by cotreatment with the PKA inhibitor H-89. PDE7A's low K_m for cAMP (0.2 μ M) and low V_{max} (0.025 nmol/min mg total protein) suggest that this enzyme may serve to maintain low cAMP levels in a restricted subcellular compartment [10].

IC242 is a potent PDE4 inhibitor that will be useful for examining PDE7 activity in vitro. However, its steroid-like structure precludes it from being a useful tool in biological settings. Additional work will be needed to develop inhibitors that can be used to establish the biological role of PDE7 in lymphoid and other tissues.

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Shaw's PDE1 mRNA expression in bone osteoblastic cells lines
↳ therefore provides a potential/plausible link to osteoporosis

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Involvement of Phosphodiesterase Isozymes in Osteoblastic Differentiation*

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HIROSHI HORIUCHI, and KUNIO TAKAOKA

ABSTRACT

The cyclic nucleoside monophosphates (cyclic adenosine monophosphate [cAMP] and cyclic guanosine monophosphate [cGMP]) are found ubiquitously in mammalian cells and act as second messenger transducers to effect the intracellular actions of a variety of hormones, cytokines, and neurotransmitters. In turn, these nucleosides also modulate the signal transduction processes regulated by a range of cytokines and growth factors. Previously, we have reported that pentoxifylline, a nonselective phosphodiesterase (PDE) inhibitor, can promote osteoblastic differentiation by elevating intracellular cAMP levels and, consequently, enhance bone formation *in vivo* and *in vitro*. In this study, we examined the expression of PDE isozymes in ST2 cells, a model of osteoblastic precursor cells, and MC3T3-E1 cells, a model of osteoblastic terminally differentiated cells.

We examined the effect of selective inhibitors for a respective PDE isozyme on the capacity of bone morphogenetic protein 4 (BMP-4)-induced alkaline phosphatase (ALP) activity, a cellular differentiation marker, in cells with osteogenic potential. The results indicate that selective inhibitors for PDE2, PDE3, and PDE4 enhanced the BMP-4-induced ALP activity in a dose-dependent manner in ST2 cells but not in MC3T3-E1 cells. Northern blot analysis also revealed that the selective inhibitors for PDE2, PDE3, and PDE4 enhanced the levels of expression of messenger RNAs (mRNAs) of ALP, osteocalcin (OC), and collagen type I in ST2 cells but not in MC3T3-E1 cells except for the treatment with PDE4 inhibitor. Given these data, we conclude that PDE isozymes are involved in the modulation of osteoblastic differentiation mainly at an early stage. Additionally, selective inhibitors for PDE2, PDE3, and PDE4 appear to promote the differentiation of osteogenic precursor cells toward an osteoblastic phenotype. (J Bone Miner Res 2002;17: 249–256)

Key words: phosphodiesterase isozyme, selective inhibitor for phosphodiesterase isozyme, osteoblastic differentiation, bone morphogenetic protein-4, cyclic adenosine monophosphate

INTRODUCTION

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) act as second messengers in the functional responses of various cells to hormones,

neurotransmitters, and other agents. In osteoblasts, for example, cAMP produced in response to parathyroid hormone (PTH) or parathyroid-related protein (PTHrP) regulates osteoblastic differentiation.^{1–4} There are corresponding data that show how administration of PTH or PTHrP also lead to increases in cancellous bone volume in animal models.^{5–7} The intracellular level of cAMP is regulated by G protein-coupled receptor (GPCR) and degradation is mediated by the phosphodiesterase (PDE) family of enzymes that catalyze the hydrolysis of cAMP and cGMP.^{8,9} Therefore, controlling

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the activities of these enzymes would in turn regulate the intracellular level of the second messengers.

Previously, we reported that pentoxifylline, a nonselective PDE inhibitor, had the potential to promote bone formation in neonatal mice.¹⁰⁰ The effects of pentoxifylline on osteoblastic cells were explored also in an *in vitro* system. Pentoxifylline stimulated osteoblastic differentiation of ST2 cells derived from bone marrow stromal cells and the chondrogenic differentiation of multipotential C3H10T1/2 cells. However, MC3T3-E1 cells derived from osteoblasts of murine calvaria were not affected by pentoxifylline. In cells in which pentoxifylline influenced differentiation, the effects were observed in the presence of bone morphogenetic protein 4 (BMP-4) in a dose-dependent manner.¹⁰¹

PDEs comprise a large group of structurally related isoenzymes derived from at least nine distinct genes and classified into nine groups based on their substrate specificity, selective inhibition or stimulation by cofactors, selective inhibition by standard inhibitors, and gene homology.¹⁰²

The classification is becoming more complex because of the presence of isoforms for a specific isoenzyme derived from differences in transcriptional start sites and splicing variant formation. The PDE families (PDE1 to PDE9 and their isoforms) are found in different amounts, proportions, and subcellular locations depending on the cell, tissue, and species.¹⁰³ Because of this variation, the identification of the PDE isoenzymes expressed in osteoblastic cells at different stages of differentiation is essential to understand the effects of PDEs in the bone-forming process. This is particularly important for BMP-induced bone formation, given that a nonselective PDE inhibitor pentoxifylline was clearly effective in augmenting this process.¹⁰⁰

We used two cell lines (MC3T3-E1¹⁰⁴ and ST2¹⁰⁵) as osteoblastic lineages at different stages of differentiation. MC3T3-E1 cells express the early stage of osteoblastic phenotype, and ST2 cells represent a bone marrow stromal cell with the ability to differentiate into osteoblasts.^{106,107} These cell lines have been used widely as the established model for the identification of many molecular mechanisms and under appropriate conditions become matrix-forming mineralization osteoblasts.^{108,109}

In this study, we attempted to identify by reverse-transcription polymerase chain reaction (RT-PCR) the PDE isoenzymes expressed in osteoblastic cell lines and their ability to regulate the biological action of BMP-4 with the use of selective inhibitors for PDE isoenzymes.

MATERIALS AND METHODS

Cell culture

A murine osteoblastic cell line MC3T3-E1 and a mouse bone marrow stromal cell line ST2 were obtained from the RIKEN Cell Bank (Tsukuba, Japan). These cells were incubated at a cell density of 3×10^5 cells/100-mm plastic dish. MC3T3-E1 was cultured with α -minimal essential medium (α -MEM; Gibco, Grand Island, NY, USA) and ST2 was cultured with RPMI1640 (Gibco). Both media contained 10% (vol/vol) heat-inactivated fetal bovine serum

(FBS; Gibco) and the cells were cultured at 37°C in a humidified 5% CO₂ incubator.

RNA preparation and RT-PCR

Total RNA was isolated from two cell lines and multiple tissues of mice (day 7, 6 weeks old) using RNeasy (Qiagen Co., Tokyo, Japan) according to the manufacturer's instructions. After treating with RNase-free deoxyribonuclease I (Gibco), 1 μ g of total RNA was reverse-transcribed using an RNA PCR kit (Takara Shimos Co., Shiga, Japan) according to the instruction manual. The reaction time was 30 minutes at 42°C. Aliquots of the obtained complementary DNA (cDNA) pool were subjected to PCR and amplified in a 20 μ l reaction mixture using Taq polymerase (Takara Shimos). Amplifications were performed in a Program Temp Control System (PC-500; ASTEC, Fukuoka, Japan) for 35 cycles after an initial denaturation step at 94°C for 3 minutes, denaturation at 94°C for 30 s, annealing for 30 s at the specified temperature, and extension at 72°C for 90 s, with a final extension at 72°C for 10 minutes. Positive controls and reaction mixtures lacking reverse transcriptase were used routinely as controls for each of the RNA samples. No PCR product was detected in the absence of reverse transcriptase during the RT step, indicating that the RNA preparations were free from intact genomic DNA. Amplification reactions specific for the following cDNAs were performed: PDE1A, PDE1B, PDE1C, PDE2, PDE3A, PDE3B, PDE4A, PDE4C, PDE4D, PDE5, PDE7, PDE8A, and PDE9. PDE4B, which had not been identified in mouse, and PDE6, which is expressed selectively in retina, were excluded. The PCR primer sequences are given in Table 1. Reaction products were electrophoresed to 1.5% agarose gel, and the amplified DNA fragments were visualized by ethidium bromide staining under UV light. All PCR products were subcloned and sequenced using the DNA sequencing kit (Applied Biosystems, Warrington, UK). The nucleotide sequences of the cloned PCR products were compared with the European Molecular Biology Laboratory (EMBL) and the GenBank databases.

Selective inhibitors for PDE isoenzymes

The selective PDE inhibitors whooposide (Sigma Chemical Co., St. Louis, MO, USA), erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; Sigma Chemical Co.), sulfinylurea (Sigma Chemical Co.), miliprin (Sigma Chemical Co.), zaprinast (Sigma Chemical Co.), and flunarizine were gifts from JT, Inc. (Owaka, Japan). All reagents were diluted in dimethylsulfoxide (DMSO). At concentrations of 0.2%, DMSO had no influence on cell viability (data not shown).

Assay for alkaline phosphatase activity

Both cell lines were plated at a cell density of 5×10^4 cells/well in 6-well plates. At confluence, the medium was changed to one containing various concentrations of each selective PDE inhibitor with or without conditioned medium (10%, vol/vol) from Chinese hamster ovary (CHO) cells transfected with mouse BMP-4 (mBMP-4) cDNA.¹¹⁰

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TABLE 1. OLIGONUCLEOTIDE PRIMERS USED IN THE PCR

Target cDNA	Primer sequence (5'-3')	Size of the PCR product (bp)	GeneBank accession number
PDE3A	5': TCACACTCTTCAAAATAGG 3': TGTCTACAGCTATGACATCC	300	U36649
PDE1B	5': GACGTACACCACTATGCTCG 3': TGTCTACAGCTATGACATCC	421	U01695
PDE1C	5': CACCATCAACTCATCATATC 3': GAGTATGCTTTTCTTCTCC	418	L70941
PDE2	5': AAGTCTCTGACAAATTCAC 3': TGTCTACAGCTATGACATCC	315	AA755487
PDE3A	5': GCTCTGAAAGCACTATAGC 3': GAAATCTCTTCAAAATAGG	322	AF099787
PDE3B	5': TGTCTACAGCTATGACATCC 3': GATCGACCTTAAAGATGTC	438	X95921
PDE4A	5': GCAACTTACACACCTGTCC 3': GTTCTTCTGCTAAGAGCTCC	276	AF031885
PDE4C	5': TGGTATCAGATAGGATTC 3': CCGTCTTCAAGCTTGGCCT	272	AJ119175
PDE4D	5': TGGCAGATATCTGTCAG 3': TGTCTACAGCTATGACATCC	389	AF034896
PDE5	5': TTTCTGCTCTAAGAGCAAGC 3': GGTATCTCTTCTTGAATAG	460	NM 001083.1
PDE7	5': ACTGACGGCATGACATGTAC 3': GACTCTACACCAAGTGTAC	239	U58171
PDE8	5': ACGGCTATTCTCTTCCAG 3': GACTTGGCAATGGTGCAGGTG	626	AF067886
PDE9	5': TACCATCTCAATCGGCGC 3': GGAAGACCTTGTCTACTC	311	AF068247

on culture days 1 and 4. These conditioned media were prepared by collecting media of CHO cells transfected with mBMP-4 cDNA at a density of 3×10^5 cells/100-mm plastic dish with 10% FBS-added α -MEM (Gibco) after 5-day culture. As control conditioned media, culture media from mock vector-transfected CHO cells were prepared in the same manner.

After 6 days of exposure to each selective PDE inhibitor with the BMP-4 or mock conditioned media, the osteoblastic cells were washed twice with phosphate-buffered saline (PBS; -), scraped off into 0.3 ml of 0.5% NP-40 containing 1 mM of MgCl₂ and 50 mM of Tris (pH 7.5), and sonicated twice for 1 s each with a sonifier (model W-220, W.1800 Co., Kyoto, Japan). Then, the cell lysates were centrifuged for 10 minutes at 3000 rpm and the supernatants were used for the enzyme assay. Adipose phosphatase (ALP) activity was assayed with the method of Kind-King, using a test kit (Nerve Laboratories, Inc., Tokyo, Japan) with phenylphosphate as a substrate. ³²P The enzyme activity was expressed in King-Astreum (K-A) units, normalized to the protein content of the sample. The protein content was determined with the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin (BSA) as the standard.

Northern blots

Twenty micrograms of total RNA were electrophoresed in 1.0% agarose gels containing 18% formaldehyde and

transferred to Hybond-N+ membrane (Amersham Pharmacia Biotech, Arlington, UK). The membrane was prehybridized in hybridization buffer (50 mM of Tris-HCl, pH 7.5, 1 mg/ml of denatured salmon sperm DNA, 1% SDS, 1 M of NaCl, 10 mM of EDTA, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, and 0.2% BSA) for 3 h at 65°C. ALP, osteonectin (ON), osteocalcin (OC), and glyceroldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were labeled with [³²P]deoxyuridine triphosphate (dCTP) using the Rediprime Labeling Kit (TAKARA Shuzo). Hybridization was performed at 68°C overnight, and the membranes were washed three times at 68°C for 1 h with 0.1 M SSC and 0.5% SDS. The membranes were stripped using boiled distilled water containing 0.5% SDS and then rehybridized. The signals were detected with a Fuji BAS 1500 Bioimaging Analyzer (Fuji Photo Film Co., Tokyo, Japan). Quantification was performed with a Scintex Lab 98 Image Gauge (Fuji Photo Film Co.), and each value was normalized against that of the GAPDH band in the corresponding lane. Then, the normalized values were compared with the calculated fold induction.

5,4,4,5-tetramethylrhodamine-2-yl-2,5-diphenylacetate bromide assay

Both cell lines were plated at a cell density of 5×10^5 cells/well in 96-well plates and the medium was changed to one containing various concentrations of each selective PDE

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inhibitor on culture day 1 and day 4. On culture days 2, 4, and 6, the viability of the cells was monitored with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Dugdale Laboratories, Kennebunk, Maine). The MTT assay is an indirect method that measures the metabolic activity of cellular enzymes. In this assay, the quantity of formazan products as measured by the amount of 570-nm absorbance is directly proportional to the number of living cells in culture.

Statistical analysis

Data are expressed as the mean \pm SD for each group. Statistical differences among treatment groups were evaluated with analysis of Student's *t*-test. The values of *p* < 0.05 were considered to be significant.

RESULTS

Expression of mRNAs for the PDE isozymes

First, we assessed the expression of each PDE messenger RNA (mRNA) by RT-PCR in MC3T3-E1 and ST2 cells. Most of the PDE isozymes are comprised of more than one isozyme (subtype) and numerous subtype splice variants (isoforms). We designed subtype specific primer sets, which covered all subtype splice variants. Both of the cell types expressed PDE1, PDE2, PDE3, PDE4, PDE7, PDE8, and PDE9 mRNAs (Fig. 1). No detectable PDE isozyme specific to osteoblastic cells could be identified.

Effect of selective PDE inhibitors on ALP activity

The effects of the selective inhibitors for the PDE isozymes on the inhibition of ALP, an early marker of osteoblastic differentiation,²⁰ in MC3T3-E1 and ST2 cells are illustrated in Figs. 2A and 2B. ALP activity significantly increased in both MC3T3-E1 and ST2 cells by BMP-4 as previously reported.^{24,25} Treatment of ST2 cells with ERNA (PDE2 inhibitor), enflumate (PDE3 inhibitor), and rolipram (PDE4 inhibitor) enhanced the BMP-4-induced ALP activity in a dose-dependent manner. The enhancement of ALP activity without BMP-4 was observed only in the treatment of rolipram at concentrations 10 μ M (1.4-fold above control). Although expression of PDE1, PDE7 (inhibitor not available), PDE8, and PDE9 were confirmed by RT-PCR analysis in ST2 cells, treatment with vinpocetine (PDE1 inhibitor), dihydrotensate (PDE7 inhibitor), butyrolol (PDE8 inhibitor), and Zepanone (PDE9 and PDE10 inhibitor) did not affect the BMP-4-induced ALP activity. In contrast, the BMP-4-induced ALP activity in MC3T3-E1 cells was not influenced by any of the selective PDE inhibitors.

Effect of selective PDE inhibitors on expression of BMP-4-induced osteoblastic differentiation marker genes

Compared with the controls, treatment with ERNA, milnacipran, and rolipram increased gene expression of ALP, OP,

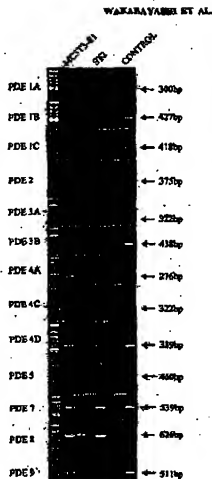


FIG. 1. RT-PCR analysis of osteoblastic lineage cell mRNA using specific primers for PDE isozymes or subtypes. Total RNA was prepared from two cell lines that were sampled on sampling confounder. Then, 10 samples were analyzed with an RT-PCR procedure as described in the Materials and Methods section. Aliquot of each of the PCR reactions was electrophoresed on 1.5% agarose gels. The data shown are typical of the results of experiments performed three times using cell preparations from different samples. A positive control and each sample performed in the absence of reverse transcriptase (omit last) also are shown in the figure.

and collagen type I in ST2 cells but not in MC3T3-E1 cells, except for the treatment with rolipram. There were no appreciable changes to the expression of OC mRNA in both cell types (Figs. 3A-3C). On the other hand, there were no appreciable changes to the expression of these genes in the treatment without BMP-4 (data not shown).

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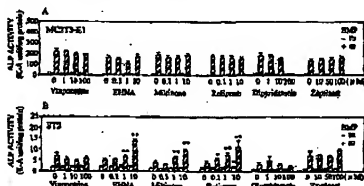


FIG. 2. Dose-response effects of selective PDE inhibitors on ALP activity in (A) MC3T3-E1 and (B) ST2 cells. ALP activity was measured as described in the Materials and Methods section. Data are means \pm SD of three replicates. Data shown are typical of the results of three different experiments. Significantly different from the control at $p < 0.05$ and $p < 0.01$. Vaniprone, PDE3 inhibitor (IC₅₀ 20 μ M); EBDA, PDE2 inhibitor (IC₅₀ 1 μ M); Mithramycin, PDE3 inhibitor (IC₅₀ 0.4 μ M); Raloxifene, PDE4 inhibitor (IC₅₀ 2 μ M); Dipyridamole, PDE3 inhibitor (IC₅₀ 9 μ M); Zalcitabine, PDE2 inhibitor (IC₅₀ 33 μ M). IC₅₀, half-maximal inhibitory concentration.

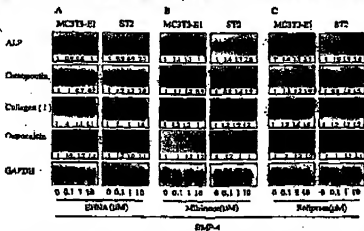


FIG. 3. Expression of osteoblastic differentiation marker genes in MC3T3-E1 and ST2 cells. Both cell lines were plated in a cell density of 20×10^4 cells/100-cm plastic dish and exposed to each selective PDE inhibitor with the EBDA conditioned media. Total RNA was isolated from control and selective PDE inhibitor-treated samples at the same time points, and the gene expression of osteoblastic differentiation marker was analyzed by Northern analysis. The effects of (A) EBDA, (B) Mithramycin, and (C) Raloxifene on the expression of ALP, Osteopontin, Collagen type I, and GAPDH. Data shown are typical of three different experiments. The numbers shown in the blots indicate the ratio of expression level between the respective mRNA and GAPDH mRNA, compared with those in the control culture.

MTT assay

Viability of cells after treatments with selective inhibitors for PDE2, PDE3, and PDE4 was monitored with the MTT assay. Only on day 2, viability of cells treated with PDE2 and PDE3 significantly increased but thereafter remained unchanged. Therefore, we concluded that none of the selective inhibitors at concentrations used in the experiments had

altered the viability of the cells throughout the culture period (Figs. 4A–4C).

DISCUSSION

This study provides evidence that osteoblastic lineage and then qualitatively expresses several PDE isozymes in the

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FIG. 4. Effect of selective PDE inhibitors on the growth of ST2 cells. Cells were cultured in 96-well plates containing various concentrations of each selective PDE inhibitor for 6 days. The number of viable cells was quantified by the MTT assay as described in the Materials and Methods section. The values \pm SD of the quadruplicate assay are shown. Data shown are typical of the results of three different experiments. (A) EENa, (B) rolipram, and (C) milrinone. Significantly different from the control at * $p < 0.05$ and ** $p < 0.01$.

mRNA level. Most of the PDE isozymes are known to comprise more than one isoenzyme product called a "PDE subtype." Furthermore, each of the PDE subtype products has one or more mRNA splice variants (PDE isoforms) resulting from either alternative splicing of mRNA or alternative transcription initiation site. Therefore, variation of PDE isozymes must be extensive and each PDE isoform might exhibit a tissue-specific distribution pattern. In this study, we examined PDE isozymes (or subtypes) but PDE isozymes specific to osteoblastic lineage were not identified. However, functional analysis involving the use of the selective inhibitors for PDE isozymes revealed that PDE2, PDE3, and PDE4 were shown to regulate the differentiation of early stage osteoblastic cells. Our findings point to a number of possibilities; either the mRNA of PDEs other than PDE2, PDE3, and PDE4 are not translated or the translated protein is either inactive, unstable,⁷⁷ or not involved in regulation of cytodifferentiation.

The PDE2 inhibitor EENa, the PDE3 inhibitor milrinone, and the PDE4 inhibitor rolipram stimulated BMP-4 action as indicated by ALP activities. Only the PDE4 inhibitor rolipram enhanced the BMP-induced expression of osteoblast markers except OC in both ST2 and MC3T3-E1 cells. Because PDE2 and PDE3 act on both cAMP and cGMP but PDE4 is a cAMP-specific PDE, the elevated intracellular level of cAMP might stimulate differentiation of both cells. Previous studies from our laboratory indicated a positive effect of rolipram on bone formation in an *in vivo* model,¹⁴⁰ and other corresponding studies were reported.^{141,142} These results indicate that PDE4 is a key functional enzyme in the regulation of BMP-4 action as an early stage of osteoblastic differentiation because the effect of ALP activity was not seen in well-differentiated MC3T3-E1 cells.

cAMP/Phosphatase Kinase A (PKA)-dependent pathways are reported to exert antiproliferative effects in osteoblasts.¹⁴³ However, regulation of differentiation by cAMP-mediated signaling through cAMP inactivation by PDEs is poorly understood in the osteoblast.^{143,144} We found that the use of the nonselective PDE inhibitor pentoxifylline induced mild accumulation of cAMP (data not shown), enhanced BMP-4-induced ALP expression *in vitro*, and stimulated systemic bone formation. This study showed that selective

inhibitors for PDE2, PDE3, and PDE4 reproduced the pentoxifylline effects in bone marrow stromal cells ST2. Furthermore, replacement of pentoxifylline by forskolin, a universal adenylyl cyclase stimulator, and by diethyl cAMP, a cAMP analog, resulted in an increase in BMP-4-induced ALP activity.¹⁴⁵ These results support the notion that signal transduction via the PKA pathway is necessary but not sufficient. Because the PDE inhibitors alone did not affect the differentiation of the ST2 cells, the effect of the PDE inhibitors or cAMP/PKA-mediated signaling is most likely mediated via BMP-4 signaling through the Smad cascade at an early stage of differentiation in osteoblasts. BMP-4 induced the expression of mRNA for core binding factor 1 (Cbfa-1),¹⁴⁶ which acts as a transcription activator in osteoblastic differentiation and a positive regulator for bone formation.^{147,148} Therefore, we examined the induction of Cbfa-1 in cells treated with the PDE inhibitor and BMP-4. However, no change in Cbfa-1 mRNA levels was observed (data not shown). A more detailed examination of this mechanism will be the subject of future studies.

The potential use of PDE inhibitors as drugs to stimulate new bone formation would be another target of this study. For this purpose, identification of osteoblast cell-specific PDE isozymes and their selective inhibitors will be required to avoid undesired side effects in patients.

Currently, BMP molecules (BMP-9) produced by DNA recombinant techniques¹⁴⁹ are ready for use in clinical practice. However, one of the issues associated with the use of these proteins is the high dose required (milligrams for 1 mm² of new bone mass) to induce new bone in humans. If some PDE inhibitors enhance BMP action, they may improve the efficacy of BMP.

In conclusion, we have found that PDE isozymes are involved in the modulation of differentiation processes in osteogenic precursor cells. Further, selective inhibitors for PDE2, PDE3, and PDE4 broadly induced osteoblastic differentiation in these cells. Further studies of osteoblastic precursors and cAMP-mediated signaling involving PDE isozymes and their corresponding inhibitors are needed. We propose that this enzyme is a potentially significant target for the development of new signal transduction pharmacotherapies for the treatment of metabolic bone disease.

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Delaware

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The First State

I, HARRIET SMITH WINDSOR, SECRETARY OF STATE OF THE STATE OF
DELAWARE, DO HEREBY CERTIFY THAT THE SAID "RINCON NETWORKS,
INC.", FILED A CERTIFICATE OF AMENDMENT, CHANGING ITS NAME TO
"SONOS, INC.", THE TWENTIETH DAY OF MAY, A.D. 2004, AT 4:01
O'CLOCK P.M.

3555387 8320

040373636



Harriet Smith Windsor

Harriet Smith Windsor, Secretary of State

AUTHENTICATION: 3125008

DATE: 05-21-04

State of Delaware
Secretary of State
Division of Corporations
Delivered 04:17 PM 05/20/2004
FILED 04:01 PM 05/20/2004
SSS 040273636 - 3555387 FILE

SECOND CERTIFICATE OF AMENDMENT
OF
CERTIFICATE OF INCORPORATION
OF
RINCON NETWORKS, INC.

(pursuant to Sections 228 and 242 of the General
Corporation Law of the State of Delaware)

Rincon Networks, Inc., a corporation organized and existing under the laws of the State of Delaware (the "Corporation"), hereby certifies as follows:

1. The original Certificate of Incorporation of this Corporation was filed with the Secretary of State of the State of Delaware on August 22, 2002.
2. A Certificate of Amendment of Certificate of Incorporation of this Corporation was filed with the Secretary of State of the State of Delaware on January 8, 2003.
3. Article I of the Certificate of Incorporation of this Corporation is hereby amended to read in its entirety as follows:

"The name of this corporation is Sonos, Inc."

4. This Certificate of Amendment has been duly adopted in accordance with the provisions of Section 242 of the General Corporation Law of the State of Delaware and the written consent of the stockholders having been given in accordance with Section 228 of such Law and written notice having been given as provided in such Section 228.

IN WITNESS WHEREOF, the Corporation has caused this Second Certificate of Amendment to be signed by its Vice President and Secretary, who hereby affirms, under penalty of perjury, that this is the act and deed of the Corporation, and that the facts stated herein are true as of May 20, 2004.

Rincon Networks, Inc.

/s/ Craig A. Shelburne
Craig A. Shelburne
Vice President and Secretary

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